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Imatinib plasma concentrations variability in hemato-oncologic patients

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pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Olivier Staub

À mes parents et à mon frère

À ma fiancée Cindy

« La philosophie, ainsi que la médecine, a beaucoup de drogues, très peu de bons remèdes et presque point de spécifiques. »

Sébastien-Roch-Nicolas de Chamfort,
In *Maximes, pensées, caractères et anecdotes*, 1795 (posth.)

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Abstract

Imatinib (Glivec®) has transformed the treatment and prognosis of chronic myeloid leukaemia (CML) and of gastrointestinal stromal tumor (GIST). However, the treatment must be taken indefinitely and is not devoid of inconvenience and toxicity. Moreover, resistance or escape from disease control occurs. Considering the large interindividual differences in the function of the enzymatic and transport systems involved in imatinib disposition, exposure to this drug can be expected to vary widely among patients. Among those known systems is a cytochrome P450 (CYP3A4) that metabolizes imatinib, the multidrug transporter P-glycoprotein (P-gp; product of the *MDR1* gene) that expels imatinib out of cells, and α_1-acid glycoprotein (AGP), a circulating protein binding imatinib in the plasma.

The aim of this observational study was to explore the influence of these covariates on imatinib pharmacokinetics (PK), to assess the interindividual variability of the PK parameters of the drug, and to evaluate whether imatinib use would benefit from a therapeutic drug monitoring (TDM) program. A total of 321 plasma concentrations were measured in 59 patients receiving imatinib, using a validated chromatographic method developed for this study (HPLC-UV). The results were analyzed by non-linear mixed effect modeling (NONMEM).

A one-compartment pharmacokinetic model with first-order absorption appropriately described the data, and a large interindividual variability was observed. The *MDR1* polymorphism 3435C>T and the CYP3A4 activity appeared to modulate the disposition of imatinib, albeit not significantly. A hyperbolic relationship between plasma AGP levels and oral clearance, as well as volume of distribution, was observed. A mechanistic approach was built up, postulating that only the unbound imatinib concentration was able to undergo first-order elimination. This approach allowed determining an average free clearance (CL_u) of 1310 l/h and a volume of distribution (Vd) of 301 l. By comparison, the total clearance determined was 14 l/h (i.e. 233 ml/min). Free clearance was affected by body weight and pathology diagnosis. The estimated variability of imatinib disposition (17% for CL_u and 66% for Vd) decreased globally about one half with the model incorporating the AGP impact. Moreover, some associations were observed between PK parameters of the free imatinib concentration and its efficacy and toxicity. Finally, the functional influence of P-gp activity has been demonstrated *in vitro* in cell cultures.

These elements are arguments to further investigate the possible usefulness of a TDM program for imatinib. It may help in individualizing the dosing regimen before overt disease progression or development of treatment toxicity, thus improving both the long-term therapeutic effectiveness and tolerability of this drug.

Résumé

L'imatinib (Glivec®) a révolutionné le traitement et le pronostic de la leucémie myéloïde chronique (LMC) et des tumeurs stromales d'origine digestive (GIST). Il s'agit toutefois d'un traitement non dénué d'inconvénients et de toxicité, et qui doit être pris indéfiniment. Par ailleurs, une résistance, ou des échappements au traitement, sont également rencontrés. Le devenir de ce médicament dans l'organisme dépend de systèmes enzymatiques et de transport connus pour présenter de grandes différences interindividuelles, et l'on peut s'attendre à ce que l'exposition à ce médicament varie largement d'un patient à l'autre. Parmi ces systèmes, on note un cytochrome P450 (le CYP3A4) métabolisant l'imatinib, la P-glycoprotéine (P-gp ; codée par le gène *MDR1*), un transporteur d'efflux expulsant le médicament hors des cellules, et l'α_1-glycoprotéine acide (AAG), une protéine circulante sur laquelle se fixe l'imatinib dans le plasma.

L'objectif de la présente étude clinique a été de déterminer l'influence de ces covariats sur la pharmacocinétique (PK) de l'imatinib, d'établir la variabilité interindividuelle des paramètres PK du médicament, et d'évaluer dans quelle mesure l'imatinib pouvait bénéficier d'un programme de suivi thérapeutique (TDM). En utilisant une méthode chromatographique développée et validée à cet effet (HPLC-UV), un total de 321 concentrations plasmatiques a été dosé chez 59 patients recevant de l'imatinib. Les résultats ont été analysés par modélisation non linéaire à effets mixtes (NONMEM).

Un modèle pharmacocinétique à un compartiment avec absorption de premier ordre a permis de décrire les données, et une grande variabilité interindividuelle a été observée. Le polymorphisme du gène *MDR1* 3435C>T et l'activité du CYP3A4 ont montré une influence, toutefois non significative, sur le devenir de l'imatinib. Une relation hyperbolique entre les taux plasmatiques d'AAG et la clairance, comme le volume de distribution, a été observée. Une approche mécanistique a donc été élaborée, postulant que seule la concentration libre subissait une élimination du premier ordre. Cette approche a permis de déterminer une clairance libre moyenne (CL_{libre}) de 1310 l/h et un volume de distribution (Vd) de 301 l. Par comparaison, la clairance totale était de 14 l/h (c.à.d. 233 ml/min). La CL_{libre} est affectée par le poids corporel et le type de pathologie. La variabilité interindividuelle estimée pour le devenir de l'imatinib (17% sur CL_{libre} et 66% sur Vd) diminuait globalement de moitié avec le modèle incorporant l'impact de l'AAG. De plus, une certaine association entre les paramètres PK de la concentration d'imatinib libre et l'efficacité et la toxicité a été observée. Finalement, l'influence fonctionnelle de l'activité de la P-gp a été démontrée *in vitro* dans des cultures cellulaires.

Ces divers éléments constituent des arguments pour étudier davantage l'utilité potentielle d'un programme de TDM appliqué à l'imatinib. Un tel suivi pourrait aider à l'individualisation des régimes posologiques avant la progression manifeste de la maladie ou l'apparition de toxicité, améliorant tant l'efficacité que la tolérabilité de ce médicament.

Résumé large public

L'imatinib (un médicament commercialisé sous le nom de Glivec®) a révolutionné le traitement et le pronostic de deux types de cancers, l'un d'origine sanguine (leucémie) et l'autre d'origine digestive. Il s'agit toutefois d'un traitement non dénué d'inconvénients et de toxicité, et qui doit être pris indéfiniment. De plus, des résistances ou des échappements au traitement sont également rencontrés. Le devenir de ce médicament dans le corps humain (dont l'étude relève de la discipline appelée pharmacocinétique) dépend de systèmes connus pour présenter de grandes différences entre les individus, et l'on peut s'attendre à ce que l'exposition à ce médicament varie largement d'un patient à l'autre. Parmi ces systèmes, l'un est responsable de la dégradation du médicament dans le foie (métabolisme), l'autre de l'expulsion du médicament hors des cellules cibles, alors que le dernier consiste en une protéine (dénommée AAG) qui transporte l'imatinib dans le sang.

L'objectif de notre étude a été de déterminer l'influence de ces différents systèmes sur le comportement pharmacocinétique de l'imatinib chez les patients, et d'étudier dans quelle mesure le devenir de ce médicament dans l'organisme variait d'un patient à l'autre. Enfin, cette étude avait pour but d'évaluer à quel point la surveillance des concentrations d'imatinib présentes dans le sang pourrait améliorer le traitement des patients cancéreux. Une telle surveillance permet en fait de connaître l'exposition effective de l'organisme au médicament (concept abrégé par le terme anglais TDM, pour *Therapeutic Drug Monitoring*).

Ce projet de recherche a d'abord nécessité la mise au point d'une méthode d'analyse pour la mesure des quantités (ou concentrations) d'imatinib présentes dans le sang. Cela nous a permis d'effectuer régulièrement des mesures chez 59 patients. Il nous a ainsi été possible de décrire le devenir du médicament dans le corps à l'aide de modèles mathématiques. Nous avons notamment pu déterminer chez ces patients la vitesse à laquelle l'imatinib est éliminé du sang et l'étendue de sa distribution dans l'organisme. Nous avons également observé chez les patients que les concentrations sanguines d'imatinib étaient très variables d'un individu à l'autre pour une même dose de médicament ingérée. Nous avons pu aussi mettre en évidence que les concentrations de la protéine AAG, sur laquelle l'imatinib se lie dans le sang, avait une grande influence sur la vitesse à laquelle le médicament est éliminé de l'organisme. Ensuite, en tenant compte des concentrations sanguines d'imatinib et de cette protéine, nous avons également pu calculer les quantités de médicament non liées à cette protéine (= libres), qui sont seules susceptibles d'avoir une activité anticancéreuse. Enfin, il a été possible d'établir qu'il existait une certaine relation entre ces concentrations, l'effet thérapeutique et la toxicité du traitement.

Tous ces éléments constituent des arguments pour approfondir encore l'étude de l'utilité d'un programme de TDM appliqué à l'imatinib. Comme chaque patient est différent, un tel suivi pourrait aider à l'ajustement des doses du médicament avant la progression manifeste de la maladie ou l'apparition de toxicité, améliorant ainsi tant son efficacité que son innocuité.

Scientific communications

Some parts of the present work have been published in international journals or presented in Swiss and international congresses as oral or poster presentations.

Publications

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Oral presentations

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Nomenclature

Abbreviations

ABL	Abelson's leukemia virus gene (in chromosome 9)
ACN	Acetonitrile
AcOEt	Ethyl acetate
AGEP	Acute generalized exanthematous pustulosis
AGP	α_1-acid glycoprotein
AL	Alkaline lysis
ALL	Acute lymphoblastic leukemia
ANOVA	Analysis of variance (used in statistics)
AUC	Area under the curve (used in pharmacokinetic modeling)
BCA	Bicinchonic acid
BCR	Breakpoint cluster region (in chromosome 22)
BCRP	Breast cancer resistance protein
bid	Twice daily
bp	Base pair (unit of length for nucleic acid sequences)
CAM	Cell-adhesion molecules
cDNA	Complementary DNA
CHUV	Centre hospitalier universitaire vaudois (i.e. Lausanne University hospital)
c-Kit	Mast/stem cell growth factor receptor (also termed CD117)
CGP	Ciba-Geigy Product
CID	Collision-induced dissociation
CL	Clearance
CML	Chronic myeloid leukemia
CNS	Central nervous system
CPT	Cell preparation tube
CRF	Case report form
CV	Coefficient of variation
CYP	Cytochrome P450
DAD	Diode array detector
DESS	Diplôme d'études supérieures spécialisées (i.e. Master of advanced studies)

DHPLC	Denaturing-High performance liquid chromatography
DMSO	Dimethyl sulfoxide
dNTP	Desoxyribo-nucleotide triphosphate
Dox	Doxorubicin
EC	Extracellular
EDTA	Ethylene-diamine-tetra-acetic acid
EGF	Epidermal growth factor
EORTC	European Organisation for Research and Treatment of Cancer
EtOH	Ethanol
FACS	Fluorescent-activated cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration (in the United States)
FISH	Fluorescence in situ hybridization
FO	First order (NONMEM estimation method)
FOCE	First order conditional estimation (NONMEM estimation method)
FORTRAN	FORmula TRANslator (a computer programming language)
GC	Gas chromatography
GF	Growth factor
GHz	Giga-hertz (speed-unit for computers' processors)
GIST	Gastrointestinal stromal tumor
GR	Grade (index of high purity for chemicals)
HBSS	Hanks' balanced salt solution
HL-60	Human leukemic cells
hOCT1	Human organic cation transporter 1 (cation uptake transporter)
HP	Hewlett-Packard® (now Agilent Technologies®)
HPLC	High performance liquid chromatography
HSP	Heat shock proteins
HUG	Hôpitaux universitaires de Genève (i.e. Geneva University hospitals)
IC	Intracellular
IC50	Inhibitory concentration 50%
id	Internal diameter (for chromatography columns)
IS	Internal standard (in analytical chemistry methods)
IU	International unit
iv	Intravenous (for drug administration)

KIT	Mast/stem cell growth factor receptor CD117 gene
LC	Liquid chromatography
LCC	Clinical Chemistry Laboratory (at CHUV)
LLC-PK1	Porcine kidney epithelial cell line
LLOQ	Lower limit of quantification
LOD	Limit of detection
Log *P*	Partition coefficient (measure of drug lipophilicity)
LOQ	Limit of quantification
MAP	Mitogen-activated protein
mAU	Milli-absorbance unit (used in UV detection)
MDR	Multi-drug resistance gene
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NADH	Nicotinamide adenine dinucleotide reduced form
NCI	National Cancer Institute (in the United States)
NH_4Ac	Ammonium acetate
NM-TRAN	NONMEM translator
NONMEM	NONlinear Mixed Effects Model
OCT	Organic cation transporter
OF	Objective function (in NONMEM, corresponding to -2 log likelihood)
p53	Protein 53 kDa (a tumor suppressor protein)
PBGD	Porphobilinogene deaminase (reference gene used to quantify cDNA amount of another gene)
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCL	Division of Clinical Pharmacology & Toxicology (at CHUV)
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
P-gp	P-glycoprotein (xenobiotic efflux transporter)
Ph	Philadelphia chromosome (landmark of CML)

PK	Pharmacokinetics
pl	Picoliter (i.e. a trillionth of liter = 10^{-12} l)
PMU	Policlinique Médicale et Universitaire (i.e. Lausanne University policlinic)
PREDPP	PREdiction for Population Pharmacokinetics (subroutine for NONMEM)
QALY	Quality-adjusted-life-years (in pharmacoeconomic studies)
qd	Daily
QD	Once a day
qMSF	Quantitative Mass Spectrometry Facility (at CHUV)
RECIST	Response evaluation criteria in solid tumors
RNAi	RNA interfering pathway
RP	Reversed phase (for chromatography columns)
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SAR	Structure activity relationships
SB	Sleeping Beauty (a synthetic transposon derived from some fish species)
SD	Standard deviation
SFSTP	Société Française des Sciences et Techniques Pharmaceutiques
si-RNA	Small interfering RNA
SLC	Solute liquid carriers
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
SPARC	Scalable Processor ARChitecture (a computer microprocessor from Sun®)
SPE	Solid-phase extraction
STI	Signal transduction inhibitor
$t_{1/2}$	Half-life of elimination
$t_{1/2a}$	Half-life of absorption
TBE	Tris/borate EDTA buffer
TDM	Therapeutic drug monitoring
TSQ	Triple stage quadrupole
UCR	Urinary cortisol ratio
Vd	Volume of distribution
VEGF	Vascular endothelial growth factor
VIS	Visible light
wt	Wild-type

Symbols

Latin letters

A	Adenine (in nucleic acid)
C	Concentration (used in pharmacokinetics); Cytosine (in nucleic acid)
d	Deuterium isotope
E	Effect (used in pharmacodynamics)
F	Forward (for PCR primers); Bioavailability (in pharmacokinetics)
G	Guanine (in nucleic acid)
h	Human
m	Mass (in mass spectrometry)
M	Molar
n	Number
p	Short arm (for chromosomes)
q	Long arm (for chromosomes)
R	Reverse (for PCR primers); Receptor
T	Temperature; Thymine (in nucleic acid)
u	Continuous variable in regression analyses
v	Volume
y	Observation used in NONMEM (typically the plasma concentration of a drug)
y	Discrete variable in logistic regression analyses
z	Fixed effect used in NONMEM (e.g. age, height)
z	Charge (in mass spectrometry)

Greek letters

α	Alpha: letter used to characterize a residue position on chemical structures
β	Beta: letter used to characterize a residue position on chemical structures
Δ	Delta: difference
ε	Epsilon: residual error of an observation *y*, also called random effect parameter
ϕ	Phi: parameters generated by NONMEM, such as clearance, function of fixed effect *z* and fixed effect parameters θ
η	Eta: interindividual error in NONMEM models
θ	Theta: fixed effects parameters generated by NONMEM
ω	Omega: standard deviation parameters generated by NONMEM

Script

Subscript

a	Absorption
d	Dissociation
i	Observation
j	Subject (i.e. individual)

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Chapter 1 General introduction

Imatinib mesylate (Glivec®), a new drug marketed in 2001, has revolutionized the treatment of two types of cancers, namely *chronic myeloid leukemia* (CML) and *gastrointestinal stromal tumor* (GIST). The present thesis focuses on a prospective pharmacokinetic study initiated in 2002 at the "Division de Pharmacologie et Toxicologie cliniques" (PCL) in Lausanne, during my Master of Advanced Studies (DESS) in Hospital pharmacy [1].

The aim of this study is to describe the population pharmacokinetics of imatinib, to identify the relevant factors likely to influence imatinib disposition, and finally to examine whether poor tolerance or resistance to imatinib treatment could be explained in some instances by alterations in its pharmacokinetics. Gaining such knowledge is determinant to evaluate if imatinib could be a candidate for a therapeutic drug monitoring (TDM) program.

This introducing chapter comprises an overview of the general concepts necessary for understanding the pathophysiology of the major cancers treated with imatinib, as well as the therapeutic options existing before and after the marketing of Glivec®. After a presentation of relevant biochemical and pharmacological informations on the drug itself, some underlying principles of drug disposition in the body will be described from a pharmacokinetic and pharmacogenetic point of view. Finally, this introduction will present a more detailed description of the aim of this work.

Various chemical, biochemical and computational analytical techniques have been used during this study. The background and theoretical informations concerning them will be given in the related chapters of this manuscript.

1.1 Cancer

1.1.1 Epidemiology

Cancer (also referred as malignant neoplasia[1]) is characterized by uncontrolled division of cells and by the ability of these cells to invade other tissues, either by direct growth into adjacent tissue (*invasion*) or by implantation into distant sites (*metastasis*). Cancer represents the second leading cause of death in the Swiss population, at the same rank as in Europe and in the United States. In

[1] A neoplasia is a disorganized cell growth in a tissue or organ, usually forming a distinct mass. A neoplasia can be benign or malignant (i.e. characterized by local invasion or metastasis, and leading *a priori* to death).

Switzerland, cancers account for 26% of all death cases, after cardiovascular diseases, which account for 40% [2]. In the United States, it is expected that the number of American citizens diagnosed with cancer each year will double in the next 50 years, notably because of the population aging. Cancer represents thus an enormous challenge to handle by physicians and scientists in the 21st century. Moreover, the social burden and costs of this disease are consequently anticipated to increase, with important socio- and pharmaco-economical implications that our health systems will have to tackle. To reduce this cancer burden, it is therefore especially important to put much emphasis on the prevention to lower all behavioral and environmental factors known to increase the cancer risks. Sustained efforts must be made so that high-quality screening services and evidence-based treatments, including pharmacotherapy, are accessible to everyone [3].

1.1.2 Biological mechanisms

After a quarter century of relatively rapid advances, cancer research has generated a rich and complex body of knowledge. For the next quarter of century, some scientists foresee that cancer research will develop into a logical science, where the complexities of the disease, described in the laboratory and clinics, will become understandable in terms of a small number of underlying principles. They forecast that one day cancer biology and treatment –at present a patchwork of cell biology, genetics, histopathology, biochemistry, immunology and pharmacology– will become an integrated science with a conceptual structure and logical coherence rivaling that of chemistry and physics [4].

This paragraph will briefly present a conceptual view of cancer mechanisms recently developed by Hanahan and Weinberg [4]. They indeed suggested that the vast catalog of cancer genotype –more than 100 distinct types of cancer have been described at present– is a manifestation of only six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1.1):

1. *Self-sufficiency in growth signals*: Normal cells require exogenous mitogenic growth signals from their environment to proliferate. On the other hand, tumor cells themselves generate many of their own growth signals. To achieve this autonomy, cells can acquire the ability to synthesize their own growth factors (GF; e.g. PDGF, platelet-derived growth factor and EGF, epidermal growth factor). GF receptors of the cells (e.g. c-Kit receptor) can also be upregulated, which makes them hyperresponsive to ambient GF levels. Finally, alterations

in components implicated in the intracellular transmission of the growth signal (e.g. Ras protein) can occur.

2. *Insensitivity to antigrowth signals*: Within a normal tissue, antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. Alterations in this process can be due to the loss of function of proteins (e.g. retinoblastoma protein) necessary to suppress mitotic progression. They can also result from the avoidance of the terminal differentiation of cells by overexpression of proteins (e.g. Myc protein) shifting the differentiation-inducing signals.
3. *Evading apoptosis*: Cell death (*apoptosis*) program is present in latent form in virtually all cell types throughout the body. Once triggered by a variety of signals, this program unfolds in precise steps. The p53 tumor suppressor protein (a DNA damage sensor) is a key component of the apoptotic signaling circuitry. Mutations involving its related gene represent the most common cause of cancer cell resistance to apoptosis.

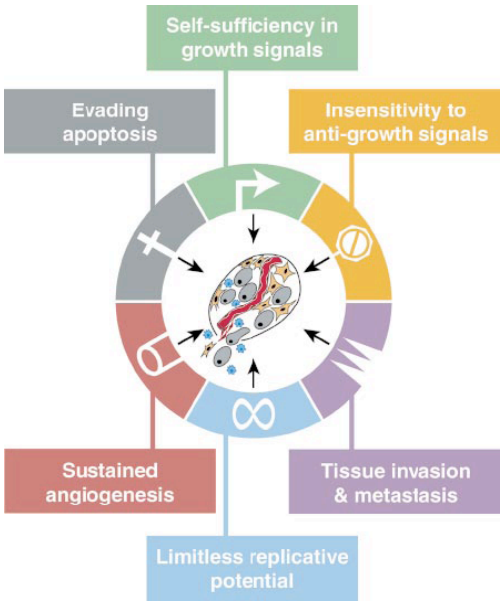

Figure 1.1. Acquired capabilities of cancer (Reprinted from Cell, 100, Hanahan D et al, The Hallmarks of Cancer, 57-70, Copyright 2000, with permission from Elsevier)

4. *Limitless replicative potential*: Even the acquisition of the three previous capabilities does not ensure expansive tumor growth to a macroscopic extent. Mammalian cells carry indeed an intrinsic, cell-autonomous program that limits their multiplication. This appears to be due to the progressive erosion of the ends of chromosomes (*telomeres*), leading to the almost inevitable cell death. Maintenance of these telomeres by telomerase enzymes provides a cause of cell immortalization when these enzymes are upregulated.

5. *Sustained angiogenesis*: The oxygen and nutrients supplied by the blood vessels are crucial for cell function and survival. The growth of these vessels in a new tissue is called *angiogenesis*. In general, the tumor cells initially lack angiogenic abilities and must develop them. The overexpression of the angiogenesis signals (e.g. VEGF, vascular endothelial growth factor), as well as the downregulation of endogenous inhibitors (e.g. interferon-β), represent strategies to activate the angiogenetic switch. Loss of the p53 upregulation function of the thrombospondin-1, a known angiogenesis inhibitor, is a well-documented example of this situation.
6. *Tissue invasion and metastasis*: Distant settlements of tumor cells (*metastasis*) are the cause of 90% human cancer death. The capability to colonize new terrains in the body allows cancer cells to reach area where nutrients and space are initially not limiting. Two main processes coexist in order to achieve such dissemination. The first one involves changes in the physical coupling of cells to their environment (e.g. alteration of CAMs, cell-cell adhesion molecules). The second one involves activation of extracellular proteases that facilitate the invasion into the tissue framework (e.g. induction of urokinase in some carcinomas).

These six capabilities should be shared in common by most and perhaps all types of human tumors. Most of them are acquired, directly or indirectly, through changes in the genomes of cancer cells. However, mutations are by themselves an inefficient process because of various DNA monitoring and repair systems that are available in our cells. It's thus rather the malfunction of these genomic "caretakers" that could explain why genomic instability leads to the three existing cancer types (epithelial, mesenchymal and hematological).

1.2 Chronic myeloid leukemia & gastrointestinal stromal tumor

1.2.1 Leukemias

Leukemias are cancers characterized by a malignant proliferation of bone marrow cells (blood cell lines or precursors). Each leukemia type may progress differently, but generally proliferative cells initially replace the normal bone marrow, interfering with its function. Malignant cells can subsequently invade other organs, with a fatal consequence if the leukemia is not treated [5]. Characterization of the first chronic leukemias was done by Bennett in 1845 [6]. Acute leukemias were discovered later in 1870. The term *leuxaimia* means "white blood" in Greek, because of the blood color of patients autopsied by Virchow in the middle of the 19th century [7].

1.2.1.1 Acute leukemias

These leukemias are characterized by an abrupt onset of clinical signs (infection, hemorrhage, pale skin) and symptoms (tiredness, arthralgia and bone pains), followed by a rapid death within a few months in the absence of treatment. They can affect adults, as well as children [5].

In this case, a minimum of 30% of all nucleus marrow cells consists of blasts (myeloid[2] or lymphoid[3] immature cells). Peripheral white cells number is typically raised, but it is also common to observe anemia and neutropenia, and generally thrombocytopenia too [5].

1.2.1.2 Chronic leukemias

Apparition of clinical signs (pale skin, spleen and/or liver hypertrophy, weight loss) and symptoms (tiredness, weakness, depression) is insidious, and death does not occur until many years after diagnosis of the disease. These leukemias mainly affect adults, but chronic myeloid leukemia forms have also been observed in children [5].

The peripheral white blood cells count is usually superior to the one observed in acute leukemias, and the bone marrow is invaded by massive amounts of immature cells. Contrary to the acute forms, the thrombocytes level is normal if not higher than normal, and thrombopenia only occurs late in the disease. On the other hand, anemia is frequently observed [5].

1.2.2 Chronic myeloid leukemia

1.2.2.1 Clinical characteristics

Chronic myeloid leukemia (CML) is a chronic myeloproliferative disorder, characterized by an important occurrence of myeloid constituent in bone marrow, peripheral blood, and other tissues [5]. Usually, a chronic phase of 3 to 5 years can be observed, followed by an accelerated phase of 6 to 18 months, and finally a blast phase (or blastic crisis) of 3 to 6 months [5,8,9]:

1. *Chronic phase*: Stage of myeloid hyperplasia with a marked leukocytosis and the presence of immature granulocytes in the blood. Thrombocytosis and, to a various extent, basophilia, neutrophilia and eosinophilia can be commonly observed at this stage. Leukemic cells are normally unable to differentiate themselves, but 50% of patients are asymptomatic at the time of diagnosis and are only identified during routine laboratory tests. Figure 1.2 shows an example of a chronic phase patient's blood film.

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[2] Myeloid cells are circulating cells produced and maturated in the bone marrow (*muelos* in Greek). These cells are either erythrocytes, thrombocytes, monocytes or polynuclear cells (i.e. neutrophils, eosinophiles, basophiles).
[3] Lymphoid cells are circulating cells maturated in the lymphatic system (i.e. lymphe node, thymus, etc). Two typical lymphoid cells are the white cells lymphocytes T and lymphocytes B.

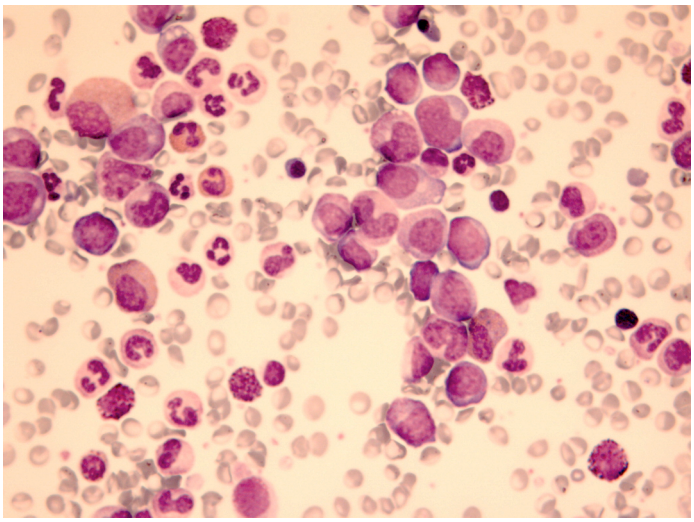

Figure 1.2. Peripheral blood film of a chronic phase CML patient (courtesy of Hematology Service, CHUV)

2. *Accelerated phase*: Intermediate stage with a progression of the disease characterized by an increase in blast cells count (15-30%) and basophiles count (>20 %), without meeting however the criteria of an acute leukemia.
3. *Blastic crisis*: Increase of myeloid immaturity and evolution to an acute leukemia.

CML can occur at any age, but is more common after the age of 45, with a median age at diagnosis close to 60 years. This disease represents 20% of all cases of leukemias and has an incidence of 1-1.5 new case for 100'000 inhabitants, each year. Before the introduction of imatinib, the average age of death was 66 years [5,9].

1.2.2.2 Molecular mechanism

Tyrosine kinases are enzymes that are able to phosphorylate (using ATP) various cellular proteins bearing tyrosine residues. Toward this end, they first have to phosphorylate themselves (autophosphorylation). In fact, a number of diseases, including cancer, diabetes, and inflammation, is linked to perturbation of protein kinase-mediated cell signaling pathways[4]. The importance of signaling in the development of cancers has already been discussed in the item no. 1 of § 1.1.2. Some of the tyrosine kinases are receptor kinases (initiating intracellular signaling) and others are cytoplasmic kinases [10]. The CML cells are characterized by the expression of an altered cytoplasmic tyrosine kinase named Bcr-Abl.

The presence of this altered tyrosine kinase is due to a reciprocal translocation between the long arms of chromosome 9 and 22. Chromosome 22 is thus shortened and named Philadelphia chromosome (Ph), which is the landmark of CML. This has been discovered as early as in 1960

[4] It is noteworthy that the human genome encodes some 518 protein kinases.

by Nowell and Hungerford [11] and provided the first proof of the association of a genetic modification with a human cancer [12]. Figure 1.3 shows the cytogenetic impact of this translocation.

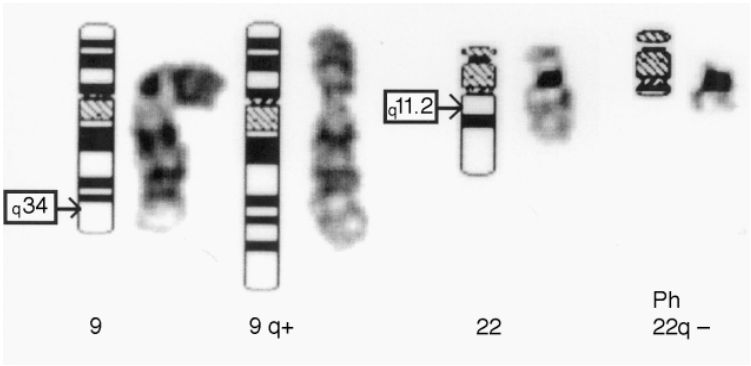

Figure 1.3. Caryotype of the t(9;22)(q34;q11) translocation, the landmark of CML (Reprinted from European Journal of Cancer, 37, Mughal TI et al, Chronic myeloid leukaemia. STI 571 magnifies the therapeutic dilemma, 561-8, Copyright 2001, with permission from Elsevier)

The molecular consequence of this translocation is the creation of an oncogene *BCR-ABL* coding for the oncoprotein Bcr-Abl. This gene results from the fusion of the oncogene *ABL* (Abelson's leukemia virus) of chromosome 9 with a sequence of chromosome 22 named "breakpoint cluster region" (*BCR*) [13]. Figure 1.4 schematizes this molecular event. Comparatively to the normal Abl protein, the Bcr-Abl protein shows an important increase of its tyrosine kinase activity. Its size depends on the break point in *BCR*, and the two most common sizes are 185 kDa (p185 Bcr-Abl) and 210 kDa (p210 Bcr-Abl). The p210 protein is found in 95% of CML patients and up to 20% of adult acute lymphoblastic leukemia (ALL) patients. Conversely, the p185 is observed in 10% of adult ALL and in the majority of pediatric Ph+-ALL patients [9].

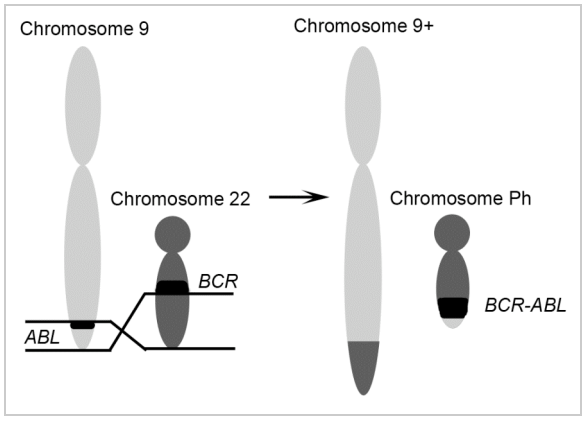

Figure 1.4. Reciprocal translocation between chromosome 9 and 22

Constitutive activation of those tyrosine kinase results in the activation of various intracellular signal transduction pathways, leading to alterations of proliferative, adhesive and survival properties of the cells, and thus to the clonal expansion characterizing cancers (see also § 1.1.2).

1.2.3 Gastrointestinal stromal tumor

1.2.3.1 Clinical characteristics

Gastrointestinal stromal tumors (GISTs) are rare tumors originating from mesenchymal stem cells of the gastrointestinal tract. Such tumors represent however the most common non-epithelial neoplasm of the gastrointestinal tract[5]. From a clinical and pathological point of view, GISTs represent a wide range of indolent tumors to aggressive malignant cancers. GISTs appear mainly in the stomach (60-70%) and subsidiarily in the intestine, or even in the rectum or esophagus. The clinical presentation is variable and the tumor is often silent before it reaches a large size. At presentation, the most common symptoms are abdominal pain and presence of a palpable abdominal mass. Many patients develop recurrent GIST, in spite of initial surgical resection. Recurrence usually occurs in the peritoneum or in the liver. After resection of the recurrent tumor, the average survival was usually only 15 months [14,15], prior to the imatinib era. Figure 1.5 shows the positron emission tomography (PET) of a patient with a metastatic GIST prior to (left-hand picture) and after (right-hand picture) a successful imatinib treatment [16].

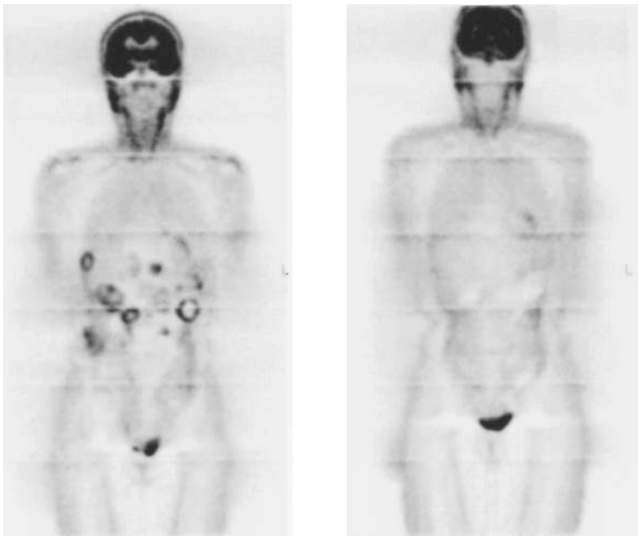

Figure 1.5. PET-scan of a metastatic GIST before and after treatment with imatinib (Copyright 2001 Massachusetts Medical Society. All rights reserved. Reproduced with permission from The New England Journal of Medicine, 344, Joensuu H et al, Brief report: effect of the tyrosine kinase Inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor, 1052-6)

[5] GIST is in fact a type of sarcoma (i.e. a cancer of connective or supportive tissue) and represents 5% of all cancer in this class.

GISTs are characterized by a highest incidence in the 5th to 7th decades, with a median occurrence age of 58. Fortunately, incidence is rather low with about 0.3 new cases per year for 100'000 inhabitants.

1.2.3.2 Molecular mechanism

Constitutive activation of the tyrosine kinase receptor c-Kit (CD117) is a key event in the pathogenesis of most GISTs. It usually results from point mutations in extra- or intracellular domains of this receptor. From a cytogenetic point of view, these mutations are often accompanied by a deletion of chromosome 14 or 22, or by a 1p deletion [17]. However, they can also occur with a normal caryotype and only point mutations. In mice, for instance, deletion of the valine in c-Kit position 558 is sufficient to induce GIST [18]. The most common site of *KIT* (i.e. coding gene of c-Kit) mutation is located at the 5' end of the exon 11, which encodes the juxtamembrane domain. *KIT* exon 9 mutation occurs in 10-15% of patients, defining a distinct subset of GISTs that are often located in the small bowel and have an aggressive clinical behavior [19]. These mutations on c-Kit could possibly be responsible *per se* for the neoplasic process, while the biological and clinical progression of the tumor may result mainly from the cytogenetic aberrations [17]. Alternately, some GISTs are characterized by another profile of mutations (*KIT* exon 13 or 17 or *PDGFRA* exon 12 or 18 mutations) or by no detectable mutations (especially in the pediatric patients) [19].

Mutations may affect the modulation domain of the receptor, as well as its enzymatic domain itself, and confer to the receptor the ability to phosphorylate numerous proteins. This results in the activation of a number of transduction signal pathways, which control cell proliferation, apoptosis, chimiotactism and adhesion (see also § 1.1.2 for more information on signaling) [17].

1.2.4 Treatments: an overview

1.2.4.1 Chronic myeloid leukemias

Before the imatinib era, standard treatments of this cancer in chronic phase were allogenic transplant of stem cells, or the use of hydroxyurea or interferon-α [9].

1. *Hydroxyurea*: Usually used for initial cytoreductive chemotherapy, but a cytogenetic response[6] is rare.
2. *Allogenic transplant*: Only curative therapy to date, with a success rate of 70-80% for young patients (< 40 years old).

[6] A cytogenetic response corresponds to a reduction of Ph+ cells in blood or bone marrow.

3. *Interferon-α*: Standard treatment since the 1980s with a hematological response observed in 80% of patients. However, a complete cytogenetic response is observed in only 10-20% of them. Adjunction of cytarabin increases response level and its coadministration represented, despite its toxicity, the gold standard when no transplant was possible.

1.2.4.2 Gastro-intestinal stromal tumor

Surgery represents the most valued treatment for the *initial phase* of GIST. Before imatinib introduction, the available options for the *metastatic* or *unresectable stages* comprised [15]:

1. *Surgery*: When used initially the survival rate at 5 years was 54%.
2. *Adjuvant after initial surgery*: Chemo- or radiotherapy used only when the resection was at risk.
3. *Systemic chemotherapy*: Since the response rate to chemical agents is very low (<10%), it was difficult to recommend any treatment. Compounds like doxorubicin were however tried.
4. *Intraperitoneal chemotherapy*: To improve the results of peritoneal resection, locoregional administration of cisplatin and doxorubicin [20], or of mitoxanthrone [21], were used.
5. *Hepatic metastasis surgery*: Such a resection allowed a survival rate of 58% at 3 years.
6. *Chemoembolization*: Option for hepatic metastasis consisting in an occlusion of the hepatic arterial bed by polyvinyl alcohol, with or without a cytotoxic agent.
7. *Radiotherapy*: Only rarely used because of the usually diffuse localization of metastases.

Following the imatinib revolution, such therapies (especially surgery) are mainly used when resistance to imatinib occurs. However, at present, new approaches aim at preventing resistance development, even before it occurs, using surgery. Furthermore, some ongoing trials are assessing imatinib as adjuvant or neoadjuvant[7] to improve the outcome of surgery in primary resectable or metastatic GIST [22].

1.3 Imatinib and its characteristics

Imatinib (Glivec®, or Gleevec® in the USA), previously known as STI571, was rationally designed to inhibit the Bcr-Abl tyrosine kinase previously described [12]. Imatinib was also found to potently inhibit the autophosphorylation of two additional tyrosine kinases: c-Kit, involved in

[7] Adjuvant therapy refers to additional treatment, usually given *after* the main treatment (e.g. surgery) where all detectable disease has been removed, but where there remains a statistical risk of relapse. Neoadjuvant therapy, in contrast, is given *before* the main treatment.

the oncogenesis of GIST [23], and the platelet-derived growth factor receptor (PDGFR), involved in the pathogenesis of the hypereosinophilic syndrome [24].

This "blockbuster" drug was approved by the FDA (United States regulatory agency) for CML within an unprecedented short time of 72 days (May 10, 2001) because of the impressive results obtained during the initial phase I and II clinical trials [8], with an impressive 90% complete response rate [25]. A phase III trial confirmed the very high percentage responses induced by imatinib in chronic phase CML [26]. However, these studies showed less impressive responses with patients in accelerated phase and blastic crisis [12]. In addition, other clinical trials have also showed an important anti-tumor activity of imatinib for GIST and the hypereosinophilic syndrome [23,24]. The drug was thus approved in the USA on February 1st, 2002 for the treatment of recurrent GIST. Swissmedic also quickly approved the drug in Switzerland for CML and GIST. For the latter cancer, imatinib was even accepted on an orphaned drug status. Remarkably, the 2-year survival of patients with metastatic GIST is now about 70% [27]. This led the chief executive officer of Novartis (the manufacturer of Glivec®) to name this drug a "magic cancer bullet[8]" [28].

1.3.1 Imatinib in the general context of cancer chemotherapy [29,30]

Imatinib constitutes one of the first examples of what is considered now as the revolution of targeted-therapy of cancer. As such, it is therefore certainly of interest to put imatinib on the more general historical context of anticancer drugs development [29]. Figure 1.6 presents a schematic view of the timeline of this history, beginning indeed in the early 1940s.

In 1942, Goodman and Gilman, two pharmacologists working for the U.S. Department of Defense, discovered that nitrogen mustards –deriving from toxics developed for chemical warfare– were able to induce tumor regression by forming alkylated intermediates covalently bonding double stranded DNA. Some years later, Farber, a pathologist at the Boston Children Hospital, discovered the benefit of antifolates (e.g. methotrexate; acting by inhibiting pyrimidines and purines synthesis) in acute lymphoblastic leukemia (ALL), and in various epithelial cancers. Afterwards, other antileukemic drugs came to clinical trials in the 1950s (especially 6-mercaptopurine and *Vinca* alkaloids) and the National Cancer Institute (NCI) began in 1955 a program for systematic drug screening. Most of the pharmaceutical companies were indeed not interested in developing anticancer drugs at that time. Then, in 1956, a combination of several

[8] The term "magic bullet" was used by the bacteriologist Paul Ehrlich to describe his goal: a specific cure for syphilis which would attack the syphilis spirochaete while having no effect whatsoever on human tissue. It is still used today to characterize wished-for drugs.

agents proved to be efficient in ALL and launched the concept of *combination chemotherapy* (like in antibacterial therapy). In 1975, a combination of an alkylating agent and 5-fluorouracil (a modestly effective drug inhibiting DNA synthesis) proved to extend survival when used after surgical resection of breast tumors. This observation initiated the concept of *adjuvant chemotherapy* (see note 7 on page 10). In 1978, the FDA approved cisplatin (a compound inhibiting DNA synthesis by covalent bounding) for the treatment of ovarian cancer, a drug also effective against a wide variety of solid tumors. Many years before, in 1956, Zubord, working for the NCI, discovered paclitaxel, a mitotic spindle poison, in the Pacific Yew tree (*Taxus brevifolia* L.). It was however not until 1992 that the FDA approved this compound, which then became as Taxol® the first "blockbuster" oncology drug.

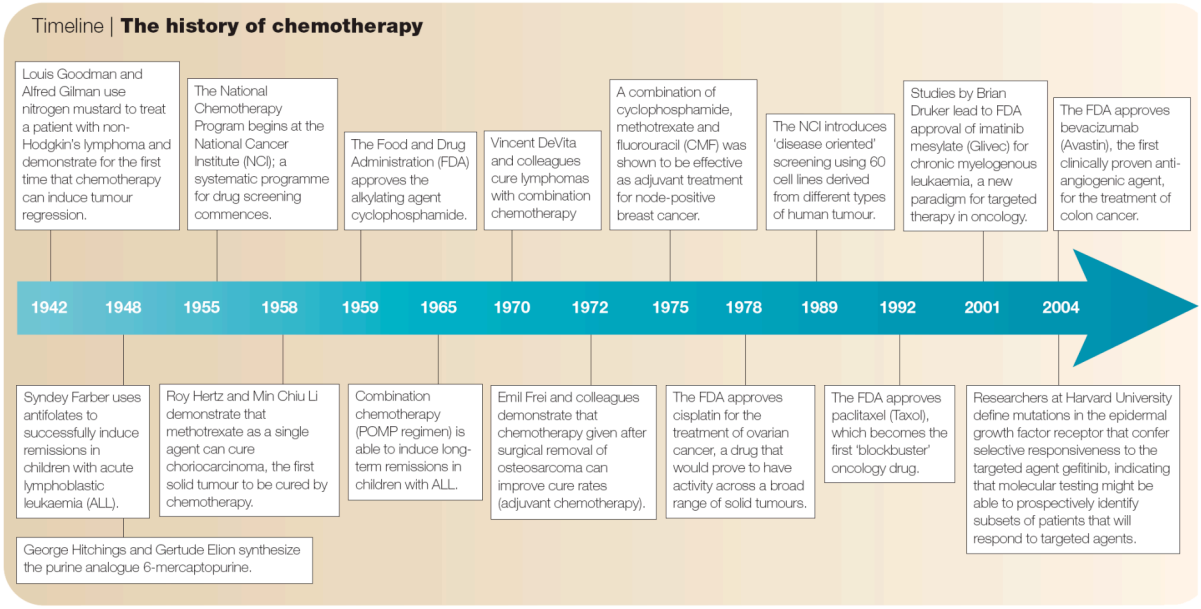

Figure 1.6. Timeline of the history of chemotherapy (Reproduced with permission from Nature Reviews Cancer, 5, Chabner BA et al, Chemotherapy and the war on cancer, 65-72, Copyright 2005 Macmillan Magazines Ltd.)

Following all these developments of cytotoxic drugs, a revolution occurred in the late 1980s. Molecular and genetic approaches uncovered entirely new signaling networks regulating cellular activities, as important as proliferation and survival. Researchers in small biotechnology firms set out to repair these molecular defects and thus initiated the era of "targeted therapy". Most of the new targets are related to the mechanisms that have been presented in § 1.1.2 (e.g. growth factors, signaling molecules, apoptosis modulators and angiogenesis promoters). In the early 1990s, this transformed cancer drug development from a low-budget government research into a multi-billion research industry, using combinatorial chemistry, high throughput screening, and

high tech *in silico*[9] methods (e.g. SAR, structure-activity relationships methods). Among the properties that the drug would ideally possess, attention was given to greater specificity to the target it was designed to, and to oral bioavailability. In that perspective, one of the landmark events in this revolution has been the development by Novartis (formerly Ciba-Geigy) of imatinib, a relatively simple structure possessing apparently all the desired features of the "ideal" targeted compound. Additional first-generation signal transduction inhibitors comprise those interfering with EGF receptor (gefitinib, inhibiting the ATP-binding function of the receptor, or cetuximab, a monoclonal antibody against the receptor extracellular domain). A third important class of compounds that is being developed consists of antiangiogenic molecules that inhibit the VEGF receptor (e.g. sunitinib, also targeting c-Kit receptor) or anti-VEGF antibodies (bevacizumab).

The next decade will probably present the challenge of designing trials aiming at combining targeted drugs and cytotoxics in a more effective manner to better treat oncologic patients. However, the pharmacoeconomic challenge of paying for all of these new agents (395 new agents are in development…) will certainly become an important issue. The total worldwide market for oncology products was approximately 46.5 billions Swiss francs in 2003, representing almost 10% of the total market of pharmaceutical, and is projected to exceed 75 billions by 2008 [29]. All these important pharmacoeconomical issues will thus have to be considered with the emergence of this targeted-therapy of cancer, but they certainly lie beyond the scope of the present introduction.

1.3.2 Development of imatinib

Back to imatinib, it is worth mentioning that the first tyrosine kinase inhibitors, tyrphostins, were already described as early as in 1988 [31]. Later on, researchers at Ciba-Geigy, under the direction of Lydon and Matter, identified a new lead compound by the screening of protein kinase C inhibitors. This compound, a 2-phenylaminopyrimidin derivative, was subsequently optimized by structure-activity relationships [9,32]. This resulted (1992) in the synthesis of the best compound with respect to activity, selectivity, and bioavailability [12]. It was initially named CGP57148B (CGP for Ciba-Geigy Product) and then STI571 (STI for Signal Transduction Inhibitor). It is chemically designed as 4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]phényl]benzamide methanesulfonate (Figure 1.7).

[9] The term *in silico* applies to computer simulations that model natural or laboratory processes (in all the natural sciences). It usually does not refer to calculations done by computer generically.

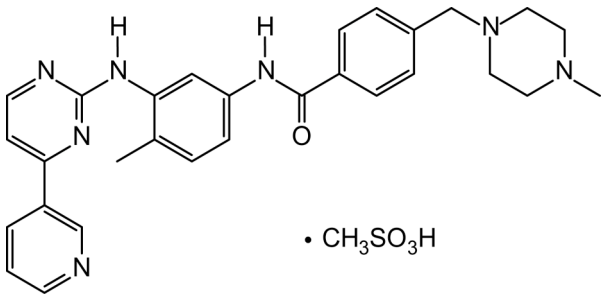

Figure 1.7. Chemical structure of imatinib mesylate

Imatinib was initially marketed as 100-mg capsules. More recently, 100- and 400-mg tablets have also been made available, with an attested bioequivalence [33]. The recommended dosing regimen is 400 mg daily for chronic phase CML patients, 600 mg daily for accelerated and blast phases CML patients, and 400 mg for GIST patients. The dose can be raised in case of lack of efficacy (if no side effects are observed) [34].

1.3.3 Pharmacodynamics

1.3.3.1 Chronic myeloid leukemia

In the general situation, tyrosine kinase inhibitors bind directly to the catalytic site of the target enzyme. Selective inhibitors, such as imatinib, occupy the catalytic site, recognizing a limited sequence variation near the ATP-binding site, as well as conformational differences between active and inactive kinases. Furthermore, imatinib binding to the enzyme also decreases its ATP binding capacity [35]. Figure 1.8 presents a schematic view of the mechanism concerning imatinib and Bcr-Abl.

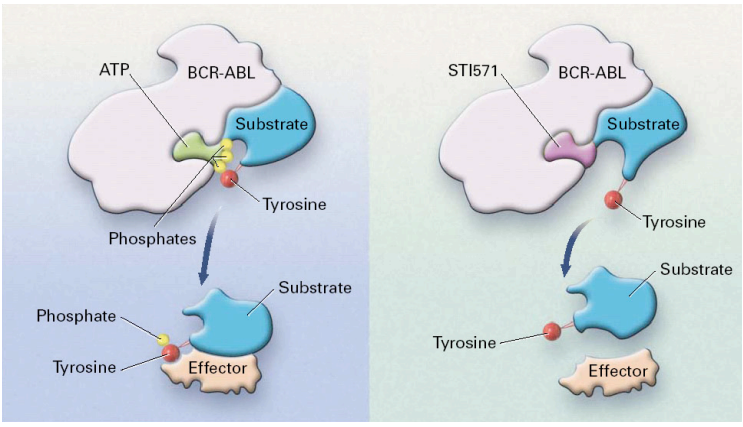

Figure 1.8. Proposed mechanism of action of imatinib: Bcr-Abl phosphorylation of a substrate in the absence (left panel) or presence (right panel) of imatinib (Copyright 2001 Massachusetts Medical Society. All rights reserved. Reproduced with permission from The New England Journal of Medicine, 344, Goldman JM et al, Targeting the BCR-ABL tyrosine kinase in chronic myeloid leukemia, 1084-6)

Imatinib captures in fact a specific inactive conformation of the activation loop of Bcr-Abl. It mimics ATP, but blocks the conformational change of the protein normally required for substrate phosphorylation [36]. Figure 1.9 depicts imatinib bound in the Abl moiety of the enzyme and freezing the kinase in its inactive conformation.

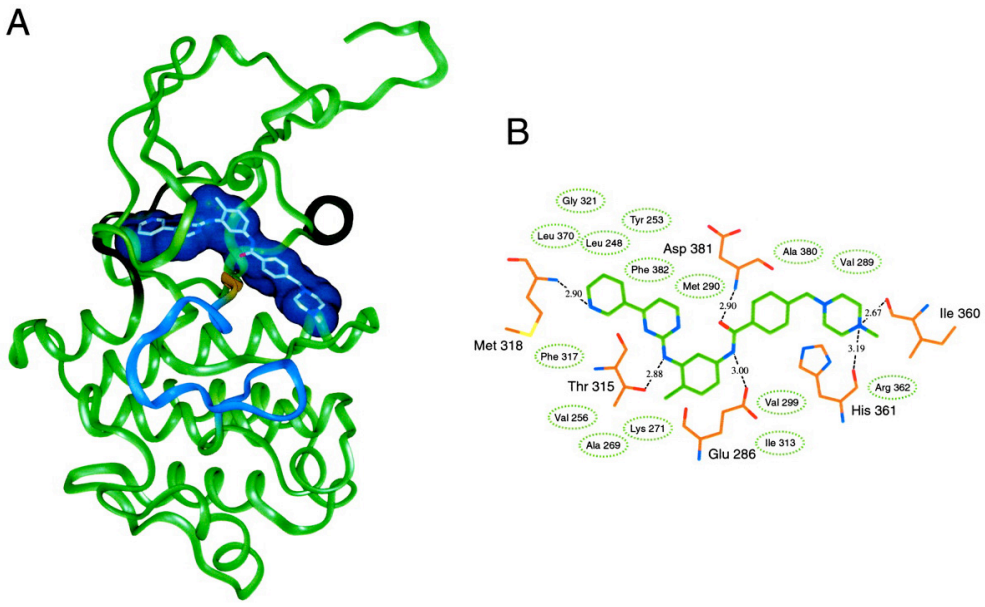

Figure 1.9. Structure of the Abl kinase domain in complex with imatinib (A: ribbon representation, with the activation loop and the van der Waals surfaces corresponding to the inhibitor colored blue; B: schematic diagram of the interactions made by imatinib, with protein residues labeled and shown in stick representation) (Reprinted from Cancer Research, 62, Nagar B et al, Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571), Copyright 2002, with permission from the American Association for Cancer Research)

By altering the binding affinity of Bcr-Abl for ATP and substrate proteins, imatinib prevents the subsequent phosphorylation of a number of cellular proteins [37]. This results in an inhibition of the constitutive activation of several proteins involved in signal transduction pathways, thus blocking the proliferation and clonal expansion of cancer cells [38].

1.3.3.2 Gastro-intestinal stromal tumors

Inhibition of the c-Kit oncoprotein by imatinib was also hypothesized, and in fact verified with the knowledge that the structure of the catalytic domain of this protein is very close to that of PDGFR, another target of imatinib [39]. The drug thus appeared to bind to the intracellular tyrosine kinase catalytic site of c-Kit by a mechanism similar to that of Bcr-Abl.

Modulation of this pathway has been experimentally demonstrated to significantly inhibit the growth of c-Kit expressing cells [39].

1.3.4 Pharmacokinetics

1.3.4.1 Absorption

Upon oral administration, imatinib is well absorbed and its bioavailability is about 98%. The maximum plasma concentration (C_{max}) is reached after 2 to 4 h [8].

1.3.4.2 Distribution

At clinically relevant concentrations, plasma protein binding is about 95%, mainly to α_1-acid glycoprotein (AGP), as well as to albumin [8,40]. The typical volume of distribution (Vd) of 295 l [41] expresses an affinity of imatinib for the tissular constituents beyond the average affinity for plasma components.

Of importance, imatinib was found to have a limited penetration into some profound body compartments, and this may have important clinical consequences. A recent study on mice has shown that the central nervous system (CNS) becomes a sanctuary site when animal harboring *BCR-ABL* transfected hematopoietic cells are maintained on imatinib for several months. Imatinib has been reported to cross only marginally the blood-brain barrier (possibly because of the high levels of P-gp in the blood-brain barrier; see § 1.4.3) [42]. This low penetration in the brain compartment was indeed found to influence the therapeutic response of a CML patient who unexpectedly experienced a blastic crisis of the CNS, although having achieved a complete cytological remission in his bone marrow. The levels of imatinib and of its active metabolite in this patient were 40-fold lower in the cerebrospinal fluid than in plasma [43].

1.3.4.3 Elimination

Imatinib is mainly metabolized by cytochrome P450 isoenzyme 3A4 (CYP3A4), and its elimination half-life is about 14 to 18 h. Its main metabolite, N-desmethylimatinib (or CGP74588) is still active with a similar *in vitro* potency and a half-life of about 20 to 40 h. It represents however no more than 10% of the dose [8,44,45] after a single oral dose and no more than 20% at steady state [42].

The drug is also metabolized, to a minor extent, by the CYP isoenzymes 1A2, 2D6, 2C9 and 2C19 [46]. Alternately, imatinib is a weak CYP2C9, 2C19 and 3A4 inhibitor [34] while its main metabolite is an inhibitor of CYP2D6 [8].

The mean total clearance (CL) value of about 10 l/h determined to date [8,47,48], and associated with the bioavailability of almost 100 %, means that imatinib is not a drug

characterized by a high extraction factor. The determinant factors for its elimination are thus the intrinsic hepatic clearance and the free fraction.

1.3.5 Safety

Even if imatinib was initially considered as a "magic bullet", it has been observed that most patients experienced adverse drug events during the various clinical trials. For CML-, as for GIST-patients, the most frequent adverse events reported are fluid retention, nausea, skin rash, asthenia and muscle cramps, with an incidence of more than 50% (grades 1 to 4). There is a trend suggesting increased incidence of grade 3/4 adverse events during the advancing phase of the disease, but this rarely leads to a discontinuation of the therapy [8,49]. However, the severity of the adverse drug reactions encountered with imatinib is generally not comparable with those usually observed with the standard cytotoxic chemotherapy agents.

1.3.6 Label-use

In Switzerland, imatinib has received the Swissmedic approval for [34]:

1. Treatment of Ph+-CML patients in chronic, accelerated and blast phases (in adults and children).
2. Treatment of malignant non-resectable or metastatic-GIST (in adults).

It is noteworthy that Glivec® therapy constitutes an oncologic treatment that must be taken indefinitely. Even in good responders, interruption of the treatment cause a relapse within only a few weeks [50].

1.4 Mechanism of resistance to imatinib

Despite the impressive efficacy identified in the initial clinical trials, resistance to imatinib develops in variable proportions of patients, especially for CML in accelerated or blast phase [12,51], and in up to 20% of GIST patients [52]. Bcr-Abl signal reactivation appears to represent the principal cause of acquired resistance to imatinib in CML patients. In 2002, Nimmanapali *et al* published a review of all potential resistance mechanisms in CML [51]. These mechanisms are summarized in Table 1.1.

Table 1.1. Mechanisms of resistance to imatinib in CML

Leukemic cells modification
Bcr-Abl dependent
Mutations in Bcr-Abl (T315I, E255K, M351T, G250E, F317I)
Bcr-Abl amplification
Bcr-Abl independent
Other mutations
Signal perennisation by Lyn and MAP kinases
Drug efflux by P-glycoprotein
Host variability
α_1-acid glycoprotein binding
Metabolism (e.g. CYP3A4)

The next paragraphs will briefly describe some important aspects concerning the mechanisms of resistance to imatinib. Focusing on this specific issue is certainly relevant in the context of our efforts to better understand the relationships between imatinib plasma levels and resistance to treatment.

1.4.1 Point mutations in Bcr-Abl and c-Kit

In August 2001, Sawyer *et al* have reported the identification of a point mutation in six of the nine patients subject to a relapse after imatinib treatment, and a gene overexpression in the three other patients. This mutation was a substitution of threonin by isoleucin in position 315 of the ATP-binding site of the kinase Abl domain (T315I; see Figure 1.9 for the exact position), at the exact position where imatinib binds [53]. All patients had previously presented a complete remission during 2 to 6 months. Since then, several other mutations have been described in resistant patients (more than 20 indeed, among more than 30 described overall [32]). It is noteworthy that *BCR-ABL* mutations have been reported to pre-exist to the onset of treatment. Imatinib treatment thus appears to select the mutant clones [54].

The mechanisms given in Table 1.1 have been identified in cell cultures while clinical resistance (i.e. relapses) reported in the literature concerned mainly patients in blast crisis [12,51]. This could in fact be expected considering the initial low response rate to the treatment observed in these patients [55].

In § 1.2.3.2, we have reported that there were two main types of GIST, those with *KIT* exon 11 mutations and those with exon 9 mutations or wild-type *KIT*. Recent data suggest that tumors characterized by an exon 11 mutation may benefit from a better response to imatinib compared to the other subset of tumors [56]. On the other hand, the mechanism of acquired resistance (i.e. arising during the course of imatinib treatment) is however less well defined than in CML.

In 2003, Weisberg and Griffin have published a comprehensive review of this field reporting some specific mechanisms of resistance in GIST patients characterized by c-Kit and PDGFR point mutations. [57]. Last year, Antonescu *et al* have also shown that about half of the imatinib-resistant GIST patients they studied presented acquired mutations in the kinase domain of c-Kit [19]. All these mutations appeared in patients having a primary *KIT* exon 11 mutation and were all substitutions located in the first (exon 13 or 14) or especially second (exon 17) *KIT* kinase domain. It is however still unclear whether a pre-existing clone is responsible for such resistances.

1.4.2 Amplification of the *BCR-ABL* gene

Several articles have addressed the important issue of *BCR-ABL* and *KIT* mutations, and their influence on the cellular resistance to imatinib [58-61]. In the case of *BCR-ABL* gene amplification, the extra copies of the gene overwhelm the imatinib cytostatic action by producing more enzymes than can be blocked by the agent. Thus, intracellular levels of imatinib may not be sufficient to effectively inhibit the large amount of coded Bcr-Abl enzyme present within the cell. In this case, for instance, the resistance may be possibly overridden by increasing imatinib levels at the intracellular site of action through a modulation of systemic and/or intracellular concentrations. Indeed, a recent study involving 54 CML patients, who were refractory to imatinib, or who had relapsed since first receiving standard dose of imatinib, was published. It showed that 65% of the subjects who had hematological resistance or relapse achieved a complete or partial hematological response when administered higher-than-conventional doses of imatinib [62].

1.4.3 Role of cell transporters

1.4.3.1 P-glycoprotein (P-gp)

Drug transporters are increasingly recognized as another key determinant of drug disposition and response [63-65]. The P-glycoprotein (P-gp) transporter is a 170-kDa transmembrane protein (shown in Figure 1.10), which is the product of the Multiple Drug Resistance gene *MDR1* (named at present *ABCB1*[10] [66]). P-gp and related multiple-resistance protein-1 (MRP-1) are ATP-binding cassette carriers that expel anticancer (as well as various other drugs or environmental xenobiotics) out of cells. They thus lower intracellular concentrations, conferring or increasing resistance to these agents. Physiologically, P-gp functions as an efflux pump

[10] ATP-binding cassette (ABC) transporter genes are a superfamily of genes encoding transmembrane proteins that function in the transport across cell membranes of a wide variety of substrates (lipids, ions, drugs, etc).

expressed in various tissues and compartment interfaces, like the blood-gut barrier, the blood-brain barrier and the blood-testes barrier. Its main purpose is to protect the whole organism and sensitive tissues from potentially harmful xenobiotics.

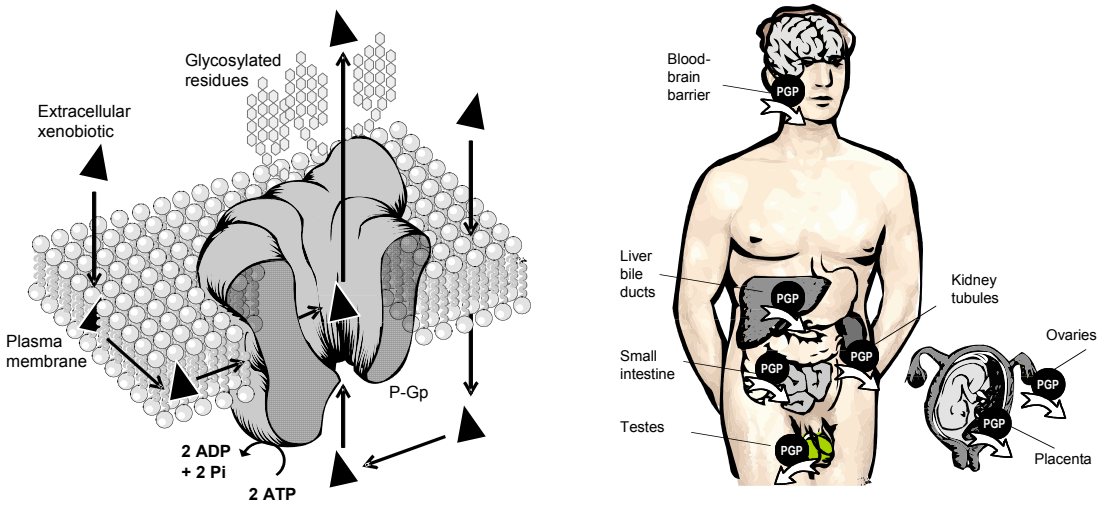

Figure 1.10. P-glycoprotein function and tissue distribution (courtesy of Buclin T)

1.4.3.2 Human organic cation transporter 1 (hOCT1)

A range of broad-specificity transporters is present in various compartments of the body for the elimination of environmental toxins and metabolic waste products. They are generally present in the liver, kidney, and intestine. Among them, the organic cation transporters OCT1, 2, and 3 mediate the facilitated transport of a variety of structurally diverse organic cations, including many drugs, toxins, and endogenous compounds. They belong to the superfamily of solute liquid carriers (SLC). OCT1 and OCT2 are mainly found in the basolateral membrane of hepatocytes, enterocytes, and renal proximal tubular cells. OCT3 has a more widespread tissue distribution, and is especially responsible for the peripheral elimination of monoamine neurotransmitters [67].

1.4.3.3 Imatinib and P-gp

Since the initiation of the present study in summer 2002, various authors have been able to demonstrate that imatinib is a substrate of P-gp [12,68], and that some aspects of resistance to imatinib treatment may therefore be caused by an increase in P-gp activity [69,70]. We indeed confirmed that P-gp expression has a marked impact on imatinib intracellular concentrations (see Chapter 6) [71].

Thus, imatinib efflux from cancer cells by the drug transporter P-gp is now seriously considered among the various mechanisms of resistance to therapy. Its clinical effectiveness may be significantly hampered in cells over-expressing P-gp. This seems to be particularly the case of

CML in blast phase [69,72]. Previous studies have demonstrated the emergence of intrinsic resistance *in vitro* (i.e. amplification of the *BCR-ABL* gene) on exposure of cells to increasing concentrations of imatinib. This indicates that the equilibrium of extra- and intracellular imatinib concentrations certainly plays a critical role in the emergence of a resistant clone [73,74].

As already mentioned, most published reports on imatinib resistance mainly focused on resistance mediated by point mutations of *BCR-ABL* [73,74]. However, the conditions favoring the occurrence of mutations are largely unknown. Several mutations in the tyrosine kinase domain of Bcr-Abl were identified in resistant patients and were found in some instance to be present prior to the initiation of the imatinib treatment [54]. Alternately, the development of cellular resistance in cells initially sensible to imatinib may be caused by P-gp efflux, which reduces intracellular disposition to levels that would not allow to completely block the replication of cells harboring *BCR-ABL*. These cells may subsequently undergo mutations, thus favoring the selection of resistant clones. In other words, P-gp may indirectly participate in the selection of cell clones harboring *BCR-ABL* mutation and amplification. Moreover, the fact that P-gp may be responsible for the appearance of cellular resistance to imatinib offers the opportunity of a pharmacological modulation of this transport system. It has been shown that doxorubicin-resistant K562 cells, which are resistant to doxorubicin through overexpression of the *MDR1* gene, are cross-resistant to imatinib, but regain imatinib sensitivity in the presence of the P-gp inhibitors verapamil or valspodar (PSC833) [70].

P-gp is not the only transporter involved in the efflux of imatinib. Two research groups have indeed recently shown that breast cancer resistance protein (BCRP, *ABCG2*) may play an important role in imatinib disposition [75,76]. By contrast, Houghton *et al* have reported that imatinib transport was unaltered between *ABCG2* and non-*ABCG2* expressing cells, rather suggesting that imatinib inhibits the function of BCRP, but was perhaps not a substrate of this transporter [77].

1.4.3.4 Imatinib and hOCT1

A recent study indicates that active influx-transport process also occurs and does affect imatinib exposure in leukemia cells. Imatinib has been demonstrated to be a substrate of hOCT1 (but not hOCT2 and 3) [78]. This suggests that differential expression of this influx transporter in target cells may be a critical determinant of intracellular drug levels and, hence, resistance to imatinib treatment. Interestingly, it has been recently found that cellular hOCT1 expression levels were much lower in nonresponders CML patients as compared to responders, in line with the

above considerations [79]. The functional consequence of the active influx transport expression on imatinib cellular levels, and its clinical relevance certainly deserves further evaluation.

1.4.4 Variability in imatinib metabolism

As previously mentioned, imatinib is mainly metabolized by the CYP3A4 and is a weak competitive inhibitor of this cytochrome. Alternately, the simultaneous administration of CYP3A inhibitors such as itraconazole or erythromycin is able to significantly increase imatinib plasma levels, whereas CYP3A inducers (rifampicin, St John's Wort, i.e. *Hypericum perforatum* L. extracts) tend to reduce them. In a clinical study with healthy volunteers, rifampicin was found to decrease imatinib exposure, and this could potentially lead to subtherapeutic plasma concentrations of imatinib [80]. Among the cytochrome P450 isoenzymes present in the liver and in the gut wall, CYP3A4/5 are the most important isoenzymes, corresponding to approximately 30% of the total content in hepatic cytochromes. CYP3A activity is known to present a wide interindividual variability, which may be furthermore influenced by a number of inhibitory or inducing compounds present in the environment (see also § 1.5.1). Consequently, the variability in the CYP3A-mediated pharmacokinetics of imatinib may put some patients at risk of suboptimal imatinib exposure. This could increase the probability of appearance of resistant cell clones with potential consequences in term of response to anticancer treatment. Moreover, high levels of expression of CYP3A in cancer cells have been reported to be responsible for the resistance to the cytotoxic drug paclitaxel, a known CYP3A substrate [81]. However, no observations are available at present to suggest that such a mechanism is also involved in imatinib resistance.

1.4.5 Modulation of imatinib free fraction by α_1-acid glycoprotein

Other mechanisms of resistance have also been incriminated [12,68], including host-dependent factors such as modulation of imatinib binding to the plasma protein named α_1-acid glycoprotein (AGP) [51]. AGP binds imatinib *in vitro* at physiological concentrations [40], and is able to reverse imatinib inhibition of Bcr-Abl [82]. In another study, higher-than-normal levels of AGP were found in the plasma from CML patients, yet such concentrations of AGP were unable to inhibit the effect of imatinib on the growth of Ph+ cells *in vitro* [83]. Moreover, no correlation was found between the elevated AGP levels and resistance to imatinib [84]. However, patients showing high levels of AGP had a slower response to imatinib.

Total plasma concentration of imatinib comprises the AGP-bound fraction, in equilibrium with free imatinib, the latter being the only species likely to penetrate intracellularly to interact with its intracellular target and exert its pharmacological action. This suggests that total plasma

concentrations may only be an imperfect surrogate of target-site concentrations of imatinib. An interaction study was carried out in patients on imatinib who also received clindamycin, which competes with imatinib binding to AGP. Coadministration of both drugs resulted in a rapid decrease in plasma levels of imatinib. The authors explain this observation by a fast tissular distribution following displacement from AGP [85]. It has been therefore suggested that clindamycin may restore imatinib intracellular concentration and activity by avoiding the exposure to marginally active drug concentrations (which could favor the selection of resistant cells) [86]. The role of AGP in this context must however be counterbalanced by the fact that therapeutic concentrations of imatinib exceed in general the total binding capacity of this plasma carrier protein.

1.4.6 Alternative strategies to overcome imatinib resistance

Beside the above-mentioned strategies devised to overcome the resistance due to P-gp, CYP3A4 and AGP, alternate approaches are being attempted to associate imatinib with other substances to counteract some further aspects of resistance mechanisms.

Heat shock proteins (HSP) are a class of intracellular proteins, named chaperones[11], helping other altered proteins to remain functional in spite of inhibition. As Bcr-Abl is client of HSP90, regimens combining imatinib with the recent drugs that act as HSP inhibitors (i.e. geldanamycin and derivatives) are currently considered [87,88]. It is premature at present to determine whether such an approach may be translated into potential clinical applications.

Alternately, imatinib was also shown to induce another signaling pathway mediated by MAP (mitogen-activated protein) kinase. Lonafarnib is a farnesyl protein transferase inhibitor, which prevents the post-transcriptional addition of a farnesyl moiety to the Ras protein involved in this MAP pathway (see also the description of cancer mechanisms in § 1.1.2). Combination of lonafarnib and imatinib allowed to inhibit resistant cell colonies [51]. In addition, lonafarnib was found *in vitro* to be a potent P-gp inhibitor that could certainly be of interest in a regimen associating it with imatinib [89].

1.4.7 Looking beyond imatinib

Further strategies developed to overcome imatinib resistances are the use of new targeted drugs. This is particularly important since imatinib buys time for most patients rather than provides a cure. The relapse rate in CML patients after 4.5 years is about 16% overall. The new

[11] Chaperones are proteins that bind to and stabilize other proteins in order to allow their correct folding or transport.

compounds have been designed to inhibit Bcr-Abl proteins harboring the point mutations previously described to confer resistance to imatinib. The two principal drugs that are emerging at present are dasatinib (BMS354825), developed by Bristol-Myers Squibb, and nilotinib (AMN107), developed by Novartis [90,91].

Dasatinib, structurally unrelated to imatinib, is 325 times more potent than imatinib, and possesses activity against 18 Bcr-Abl mutations. At present, dasatinib demonstrates significant efficacy in both imatinib-resistant and intolerant patients (6-month data from the multicentric phase II study START-C) [91]. Alternately, nilotinib is a new derivative based on imatinib structure and inhibiting 32 mutant forms of Bcr-Abl. It is 20-fold more potent than its predecessor and shows a 60% hematological response rate in imatinib-resistant CML patients [91]. However, neither dasatinib nor nilotinib inhibit the T315I mutant form of Bcr-Abl [90].

1.5 Potential importance of pharmacokinetics and pharmacogenetics in clinical response variability of imatinib

The pharmacokinetics of many drugs considerably differs between individuals, largely because of variations in the expression of metabolizing enzymes in the liver, intestine and other tissues. In particular, the cytochrome P450 enzyme system is involved in the metabolism and elimination of almost half of the drugs on the market [92]. In addition, drug transporters such as P-glycoprotein (P-gp) are recognized as key determinants of drug disposition and response [93]. As previously detailed, the activity of these systems is different from person to person. This leads to highly variable drug elimination rates, hence to interindividual differences in circulating drug concentrations and in therapeutic response.

Pharmacogenetics is the study of genetic variations underlying differential responses to drugs. Inherited differences in the metabolism, transport and disposition of drugs, and in the drug targets, greatly affect the efficacy and toxicity of medications (Figure 1.11). The existence of genetic polymorphisms[12] of drug-metabolizing enzymes and drug-transporters proteins has thus provided an explanation as to why some patients do not obtain the expected drug effects, or show an exaggerated drug response and serious toxicity after taking standard doses of a drug [94].

[12] Polymorphisms are variations in an organism's DNA sequence, which accounts for all naturally occurring alleles (i.e. viable coding sequences of a gene).

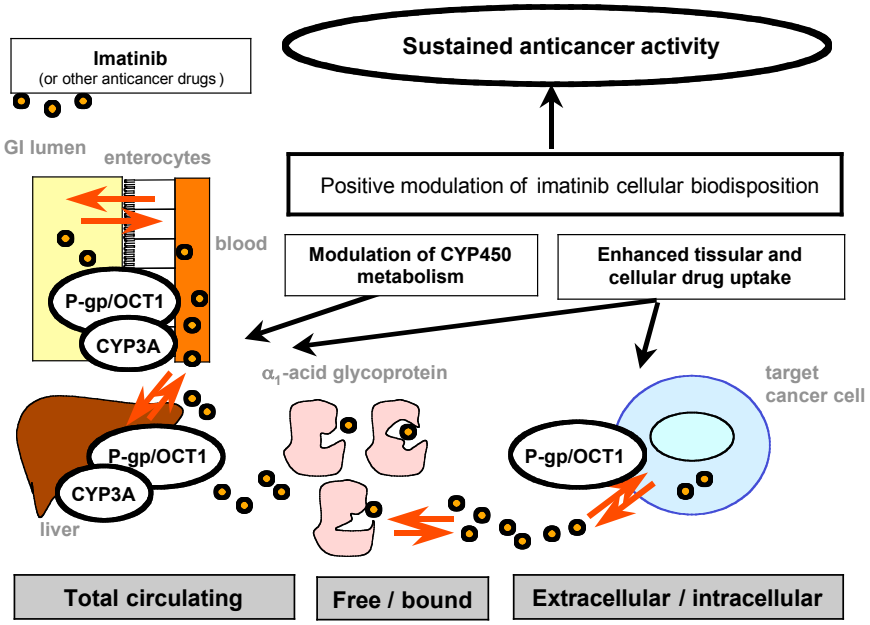

Figure 1.11. Genetic variability of CYP and P-gp affecting imatinib activity, and potential targets for PK optimization (courtesy of Decosterd LA, with modifications)

1.5.1 Pharmacogenetics of CYP3A4/5

Although no polymorphisms modifying the enzyme structure and activity have been formally identified to date, some polymorphisms are known to affect the gene promoter and hence the expression and inducibility of CYP3A4. In fact, there is roughly a 10-fold variability in CYP3A4 activity among different subjects, and this is one of the most important factors responsible for the large intra- and interindividual pharmacokinetic variability observed during drug treatments [92]. Moreover, CYP3A5, a closely related isoenzyme of CYP3A4, displays a significant polymorphism with about 75% of Caucasian subjects harboring an inefficient allele. This polymorphism has been shown to affect the disposition of CYP3A substrates [95].

The determination of the CYP3A enzymatic activity (phenotyping) in patients may be clinically useful and has been notably used in the present study. The CYP3A4/5 activity can be assessed using various tests (erythromycin breath test, plasma clearance of midazolam, 6β-hydroxycortisol/cortisol urinary ratio). Among them, the measurement of the endogenous levels of 6β-hydroxycortisol/cortisol[13] in urine (UCR) is an attractive approach, as it does not require the administration of any exogenous probe substance [96]. This non-invasive test only requires the collection of a single urinary spot, which can be analyzed by LC-MS/MS after solid-liquid phase extraction.

[13] 6β-hydroxycortisol is the product of cortisol metabolization by CYP3A4. The product to parent ratio has been therefore proposed as a marker of CYP3A4 activity.

Besides being variable, CYP3A activity may be inhibited or induced by numerous drugs, as well as by environmental and dietary substances. This results in elevated plasma levels of substrates of this enzyme such as imatinib, associated with possible adverse drug reactions, or alternately leading to a lowering of plasma levels with potential consequences on the clinical response. The phenotyping of CYP3A has the advantage of reflecting both the acquired modulation (by environmental factors) and the genetic determinant of enzyme activity.

1.5.2 Polymorphism of the *MDR1* and *hOCT1* gene

There is a large interindividual variability in the expression and activity of the drug efflux transporter P-gp, in part due to the polymorphism of the *MDR1* gene, notably the C3435T single nucleotide polymorphism (SNP) at exon 26, with functional [97,98] and clinical [99] consequences. This polymorphism has been reviewed in detail [93,100]. In childhood ALL, a 7.1% reduction of CNS relapse was observed in patients harboring the *MDR1* 3435 TT or CT genotype, associated with a reduced expression and function of P-gp [101]. Similarly, in a study performed in Lausanne, this same C3435T SNP has been found to be a factor influencing the immune recovery of HIV patients at the initiation of their antiretroviral therapy [99]. In fact, HIV protease inhibitors, like imatinib, are substrates of P-gp. Individuals with the 3435 TT genotype have low expression of P-gp in their peripheral blood mononuclear cells (PBMCs)[14] as compared to the case of wild-type 3435 CC patients. It was thus hypothesized that the *MDR1* C3435T polymorphism would influence the intracellular accumulation of P-gp substrates such as HIV protease inhibitors, thereby explaining the immunological benefits observed in HIV patients with the *MDR1* 3435 TT genotype. Indeed, it was confirmed that patients with the *MDR1* 3435 TT genotype, associated with reduced P-gp expression, had a more than 2-fold increase in PBMCs intracellular levels of the protease inhibitor nelfinavir, a P-gp substrate [66].

As far as hOCT1 is concerned, genetic polymorphic variations have also been identified, but their functional and clinical consequences have been until now the focus of less intense investigations, in comparison to *MDR1*. It seems that the transporter variants with the A61C, C88A, G220V, G401S, G465A mutations result in a reduced uptake, whereas the S14P variant results in increased uptake activity [67]. In fact, much remains to be learned from this transporter genetic.

[14] PBMCs are circulating mononuclear cells, i.e. lymphocytes and monocytes.

It thus appears that both genetic and environmental factors are likely to affect the expression and function of P-gp and hOCT1, with possible clinical consequences on the outcome of treatment with a P-gp and hOCT1 substrate such as imatinib.

1.5.3 α_1-acid glycoprotein polymorphism

Two genes arranged in tandem control the expression of AGP: *AGP1* and *AGP2*. They are closely linked on chromosome 9, and encode two ORM proteins (ORM1 and ORM2, respectively). While the ORM2 locus is monomorphic in most populations, at the ORM1 locus, three codominants alleles (*ORM1*F1, ORM1*F2* and *ORM1*S*) have been characterized. *ORM1*F1* and *ORM1*S* are observed worldwide, while *ORM1*F2* is rare and is limited to Caucasians and West Asians. Although these two proteins are generally measured at a molar ratio of about 3 : 1, the relative concentrations of the ORM variants can largely vary [102]. However, the functional implications of the relative occurrence of these alleles –either on AGP plasma levels or on drug binding– is still essentially unknown.

In the present work, the influence of the diverse genetic variants has not been considered. We only measured the total AGP plasma levels.

1.6 Population pharmacokinetics

All drugs exhibit pharmacokinetic (PK) variability to a greater or lesser extend. Population pharmacokinetics aims at describing this variability in terms of a number of factors [103].

1. *Fixed effects*: population average values of PK parameters, which may be function of various patient characteristics (demographical variables such as weight or gender; physiological variables such as creatininemia; external influences such as comedications).
2. *Random effects*: they quantify the amount of pharmacokinetic variability that is not explained by the previous effects (i.e. inter- and intraindividual variability).

Estimation of these effects allows designing dosage regimens for specific populations of patients (e.g. elderly or patients with renal impairment), and the individualization and optimization of these regimens integrated in a TDM program (see following paragraph). All new drugs, as well as all drugs for which a therapeutic range has been identified, should ideally be subjected to such an analysis. This was done for imatinib, but such a modeling was barely mentioned in literature at the time of marketing [8]. Only recently more extensive data were published on population pharmacokinetics in GIST patients [47], and in CML patients [104].

Our study thus aimed mainly at assessing further imatinib pharmacokinetics variability and its relationship to various demographical and biological covariates (especially *MDR1* polymorphism, CYP3A4 activity and AGP plasma levels). In order to achieve such an analysis, the NONMEM (which stands for NONlinear Mixed Effects Model) approach was used (see Chapter 3).

1.7 Imatinib and therapeutic drug monitoring strategy

1.7.1 Therapeutic drug monitoring

During the past decades, it has been established that the therapeutic use of selected drugs could be optimized by an individualization of their dosage, based on blood concentrations measurement. Such a feedback strategy, termed TDM (for Therapeutic Drug Monitoring), is now current practice for drugs such as digoxin, lithium, aminoglycosides, vancomycin, cyclosporine and antiepileptics [105]. In the anticancer chemotherapy, the follow-up of methotrexate plasma levels decay in patients is used for the adjustment of folinic acid dose required for the rescue [106]. More recently, monitoring of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors has also been progressively adopted in our hospital. The therapeutic benefit of the TDM of antiretrovirals on the outcome, quality of life and survival of HIV patients is currently under evaluation [107,108]. This approach seems both to increase the probability that a consistent therapeutic effect is reached in the treated patient, and to decrease the risk of toxic side-effects. For some drugs, TDM has been shown cost-effective in economic evaluations, provided that blood levels are subjected to expert interpretation [109]. At the "Centre hospitalier universitaire vaudois" (CHUV), TDM is performed by clinical pharmacologists and hospital pharmacists at the Clinical Pharmacology Division.

Generally, suitable drugs for a TDM program are those with large interindividual but low intraindividual pharmacokinetic variability, with both consistent concentration-response and concentration-toxicity relationships, and for which no easily measurable markers are available to evaluate efficacy and to precociously detect toxicity. Table 1.2 represents in details the common criteria a drug should own to benefit from a TDM approach [110,111].

Various indications exist for the measurements of drug concentrations, such as for instance a) individualizing therapy (at time of early therapy or dosage change); b) avoiding toxicity; c) monitoring drug interactions; and d) monitoring compliance [112].

Table 1.2. Components of the target concentration strategy

Analytic
An appropriate drug assay is available
Pharmacokinetic
Documented and significant interindividual variability in drug disposition
Adequate pharmacokinetic data available concerning the drug (including in the specific subpopulation considered)
Unpredictable pharmacokinetic parameters (intrinsic variability or confounding factors)
Pharmacodynamic
Pharmacological effect proportional to the plasma drug concentration
A narrow therapeutic range (i.e. range between efficacious and toxic concentration) exists
A constant pharmacological effect over an extended period of time exists
Clinical
Therapeutic range has been clinically established
No easily measurable clinical markers are available (e.g. intraocular pressure for glaucoma)
Duration of drug therapy sufficient for the patient to benefit from TDM
TDM will provide more information than sound clinical judgment only

1.7.2 Imatinib and TDM

As previously mentioned, imatinib is mainly metabolized by cytochrome P450 3A4 [8], whose enzymatic activity presents a large interindividual variability and is susceptible to induction or inhibition. Thus, a given dose of imatinib may yield circulating concentrations departing in selected patients from those expected based on the average pharmacokinetic data established in stringent phase I and II clinical studies. Imatinib could therefore be a suitable candidate for an individualization of dose prescription based on TDM. The follow-up of imatinib circulating concentrations could play an appreciable role in delaying the appearance of tumor resistance, which seems to be favored by repeated exposure to subtherapeutic drug levels of imatinib both *in vitro* [73,112] or *in vivo* [113].

Just recently, Judson *et al* have presented data indicating that imatinib clearance tends to increase (i.e. plasma levels are decreasing) over time upon Glivec® treatment. Imatinib levels in patients in which the dosage was increased (upon progression) were substantially lower than at the beginning of the treatment [47]. Conversely, a 25% decrease in imatinib clearance has been reported in another study in CML patients, albeit followed up for one month [104]. Such uncertainties on the pharmacokinetics of imatinib suggest a potential clinical interest to monitor imatinib peripheral blood levels.

The present thesis work will address only some of the steps necessary to evaluate the potential benefit of a TDM program. The analytical method development is given in Chapter 2. The pharmacokinetic evaluation is reported in Chapter 3. Some aspects of the pharmacokinetic-pharmacodynamic analysis are addressed in Chapter 4. Finally, examples of applications of imatinib plasma levels monitoring in selected clinical situations are presented in Chapter 5.

1.8 Aims of this work

Considering the above mentioned issues, the overall purpose of the work presented in this thesis has been formally to describe the population pharmacokinetics of imatinib, to identify factors likely to influence it, and finally to examine whether poor tolerance or resistance to imatinib treatment could be explained in some patients by alterations in its pharmacokinetic characteristics. The specific goal of our research study and its ancillary projects were as follows:

1.8.1 Main aims of the study

1. To study imatinib pharmacokinetic interindividual and residual intraindividual variability in CML- or GIST-patients, using a population pharmacokinetic approach.
2. To assess the influence of *MDR1* polymorphism in imatinib pharmacokinetics in this patient population.
3. To evaluate CYP3A4 enzyme activity in the same patients by measuring the urinary 6β-hydroxycortisol/cortisol ratio.
4. To determine the role of α_1-acid glycoprotein on imatinib pharmacokinetic variability in this patients' population.

1.8.2 Subsidiary aims of the study

1. To study *in vivo* the intracellular disposition of imatinib in patients.
2. To study the relationship between imatinib plasma concentrations, possibly modulated by AGP levels, and the clinical outcome (relapses, initial/secondary failure, side effects). In short, to ascertain whether relapses are more frequent in patients with low imatinib plasma concentrations, and alternately whether adverse drug reactions are more prevalent in patients with high imatinib concentrations.
3. As the PK-PD relationship may be obscured, or confounded, by the cancer genetic heterogeneity, to distinguish the importance of tumor genotypes on response to imatinib therapy.

4. As a complement to this study, to assess *in vitro* (in cellular models) the functional consequence of *MDR1* gene expression, or siRNA-mediated silencing, on intracellular concentrations of imatinib.

The full protocol of the clinical part of this study is presented in Appendices 1.1 to 1.5 and will be detailed further in Chapter 3.

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Chapter 2 Analytical methods

This chapter will provide a description of the various analytical procedures that have been developed and/or applied during the population pharmacokinetic study (see Chapter 3). Special attention will be given to the methods to which I have contributed mostly, namely the development of a HPLC-UV method used for the determination of imatinib concentrations in plasma from patients (and intracellularly for some of our *in vitro* studies). Attention will be given as well to the analysis of our patient's *MDR1* genotype (one of the biological covariate of our PK study) using a procedure available at the Clinical Chemistry Laboratory (LCC) of our hospital.

The initial development of the measurement of urinary cortisol and 6β-hydroxycortisol (a second biological covariate) by LC-MS/MS [1], as well as of the setting up of the intracellular measurements of imatinib in peripheral blood mononuclear cells (PBMCs) by LC-MS/MS [2], have been the subject of two Pharmacist diplomas presented at the Faculty of Sciences at the University of Lausanne. Application of these methods during the present study was performed by a laboratory technician at the Division of Clinical Pharmacology. The α_1-acid glycoprotein plasma levels (the third main biological covariate evaluated) were measured at the Laboratory of Clinical Biochemistry and Psychopharmacology Unit in the Cery hospital.

2.1 Determination of imatinib in human plasma by LC-UV

The setting up and the validation of the HPLC-UV method, as well as its application for the measurement of imatinib in patients' plasma samples, is the principal analytical chemistry development of this thesis work. It has been published in a peer-reviewed journal in 2004 [3], and has also been applied for the intra- and extracellular measurement of imatinib in *in vitro* cell cultures during a side-study on the functional influence of P-gp expression (see Chapter 6).

2.1.1 Theoretical introduction

2.1.1.1 Chromatography

Chromatography is a physico-chemical technique used for the separation, identification, isolation (preparative) and/or quantification (analytical) of the different chemical components of a –most often– complex mixture. These mixtures may be a pharmaceutical formulation of a drug (see for example, the quantification of captopril in an oral solution, which was developed as a part of a previous project at the hospital pharmacy of our hospital [4]), or a complex biological

matrix (i.e. patients' plasma samples, such as in the present study). Most chromatographic methods imply the use of a mobile phase, which can be a gas (GC, gas chromatography) or a liquid (LC, liquid chromatograpy) [5] distributed through a stationary –either solid or liquid– phase. The various components of a given mixture are carried out by the mobile phase through the stationary phase. Depending on their relative distribution in the mobile and stationary phase, and their respective affinity (i.e polarity, lipophilicity, affinity, and size) to the stationary phase, a difference in the delay of elution of the various components of a mixture out of the chromatographic column will occur. This difference in turn results in their physical separation, which can be exploited for their isolation and/or quantification.

2.1.1.2 High-performance liquid chromatography with UV detection (HPLC-UV)

HPLC is a chromatographic technique using a liquid mobile phase, which is distributed at high pressure (several hundred bars) through a stationary phase. This latter phase consists of a solid material contained in a stainless tube and characterized by a very high specific area (e.g. silica-derived or synthetic polymers). HPLC can be coupled with a variety of detectors, such as single MS or tandem MS (see *infra*), or spectrofluorimetry. Spectrophotometric UV detectors measure the ultraviolet or visible light absorption of compounds eluted from the chromatography column. It is also possible to couple HPLC with a UV diode array detector (DAD), which allows the online recording of the entire UV or visible spectra of analytes during their passage trough the photometer cell. This provides additional information on compounds identity and analytical method selectivity [5]. At present and because of its robustness, UV detection remains the more frequently used method in the routine practice of hospital pharmacy and clinical pharmacology laboratories. In fact, HPLC is generally considered to be the "reference" method, when alternate analytical methods are developed. However, LC-MS or LC-MS/MS technologies certainly tend to be increasingly applied in more recent analytical methods developments.

It is generally necessary to eliminate from sample as many matrix constituents as possible –likely to interfere with the analytes measurements–, prior to the sample injection into the chromatographic system. This is typically made by precipitation or extraction. A commonly used extraction method is solid-phase extraction (SPE). It consists in the purification and concentration of analytes from a complex mixture by adsorption onto a disposable solid-phase cartridge, followed by selective elution of the analyte with an appropriate solvent [6,7].

The above-mentioned techniques were applied for the measurement of imatinib concentrations in plasma from the patients participating in the present clinical study.

2.1.2 Materials and methods

2.1.2.1 Chemicals

Imatinib mesylate was kindly provided by Novartis (Basel, Switzerland). Clozapine (IS, Internal Standard) stock solution (250 mg/ml) in methanol (MeOH) was obtained by extraction with MeOH of a Leponex® (Novartis, Basel, Switzerland) tablet. This solution was diluted before use. MeOH for chromatography LiChrosolv® and ammonium acetate (NH_4Ac) GR for analysis were from E. Merck (Darmstadt, Germany). All other chemicals were of analytical grade and used as received. Ultrapure water was obtained from a Milli-Q® UF-Plus device (Millipore).

2.1.2.2 Chromatographic system

The chromatographic system consisted of a Hewlett-Packard 1050 pump connected to a spectrophotometric UV-VIS DAD 1050 detector set at 261 nm (Agilent, formerly Hewlett-Packard, Böblingen, Germany). This wavelength corresponds to the maximal absorption of imatinib under the chromatographic analysis conditions. The separation was performed at room temperature (RT) on a ChromCart® cartridge column (125 mm length x 4 mm id) filled with Nucleosil 100-5 μm C18 *AB* (Macherey-Nagel, Düren, Germany), and equipped with a guard column (8 mm length x 4 mm id) filled with the same packing material. The injection volume was 50 μl.

The HP-ChemStation A.06.03 software (Hewlett-Packard, Böblingen, Germany) was used to pilot the HPLC instrument and to process the data. Baselines were visually inspected and were manually adjusted using peak start and end features of the HP-ChemStation software.

2.1.2.3 Mobile phase solutions

Solvent A consisted of water containing 0.05% w/v of NH_4Ac and solvent B was MeOH containing also 0.05% w/v of NH_4Ac. The mobile phase was delivered at 1 ml/min and the gradient program conditions are given in Table 2.1.

Table 2.1. Gradient elution program

Time (min)	Solvent A (%)	Solvent B (%)	Flow (ml/min)
0	80	20	1.0
20	50	50	1.0
36	40	60	1.0
36.5*	0	100	2.0
40.5*	0	100	2.0
41*	80	20	1.0
45*	80	20	1.0

*Rinsing / re-equilibration program.

2.1.2.4 Stock and working solutions, plasma calibration and control samples

The stock solution of imatinib mesylate was prepared at a concentration of 1 mg/ml (calculated as base) in 100% MeOH and was appropriately diluted with 50% MeOH for the preparation of working solutions at concentrations of 2-200 μg/ml. Plasma calibration samples at 0.1, 0.3, 0.9, 2.5, 5.0, 10.0 μg/ml, together with plasma control samples at 0.2, 4.5, 9.0 μg/ml, were prepared by 1 : 20 dilution of the respective working solution to blank plasma (total added volume ≤ 10% of the biological sample volume), in accordance with the recommendations on bioanalytical method validation [8-10].

The working solutions were stored at -20°C and the calibration and control samples were prepared on the day of the analysis. The IS stock solution at 250 mg/ml was diluted to 105 μg/ml with 50% MeOH prior use.

2.1.2.5 Samples preparation

750-μl samples of plasma (calibration, control) were diluted with 200 μl H_2O and 50 μl of diluted IS solution (thus providing a clozapine plasma concentration of 7.0 μg/ml) in an Eppendorf microvial. The resulting solution was vortexed and centrifuged for 10 min in a centrifuge at 20'000 g (14'000 rpm) (Benchtop Universal 16R, Hettich, Bäch, Switzerland). Plasma samples from patients were processed similarly.

The clean-up procedure of diluted plasma samples was performed by solid-phase extraction (SPE) using a 24-tube vacuum manifold Macherey-Nagel (Düren, Germany). The C18 cartridges of 100 mg packing with 40-45 μm diameters and 60 Å porosity (Supelclean LC-18, Supelco, Buchs, Switzerland) were conditioned with 2 ml of methanol (in two 1-ml aliquots) followed by 2 x 1 ml water. An aliquot (900 μl) of the diluted plasma sample was applied onto the cartridge and drawn through completely under vacuum (ca 2.5 mm Hg).

The cartridge was washed twice with 1000 μl H_2O and a light vacuum (about 10 mm Hg) was applied thereafter for 5 min. Imatinib and clozapine were subsequently eluted with 1500 μl

methanol (in three aliquots of 500 µl), followed by a final drying step (about 10 mm Hg vacuum). The eluted solutions were evaporated to dryness under nitrogen steam at RT for approximately 45 min, and the residue was reconstituted in 180 µl of 50% methanol. The resulting solutions were carefully vortexed and centrifuged at 20'000 g for 10 min. Aliquots (80 µl) of the supernatants were introduced into 200 µl HPLC microvials (Hewlett-Packard, Böblingen, Germany) and a volume of 50 µl was injected onto the HPLC column.

2.1.2.6 Calibration curves

Quantitative analysis of imatinib was performed using the Internal Standard (IS) method. The calibration curves were obtained by 1/x weighted least-squares linear regression of the peak-area ratio of imatinib to clozapine (IS), *versus* the ratio of the injected amount of imatinib to IS, in each standard sample. The calibration was established over the range 0.1-10.0 µg/ml, where the response ratio / amount ratio was linear.

Clozapine was chosen as the internal standard because it shares some structural similarity with imatinib (heterocyclic base with a piperazinyl group). It has already been used as an IS for the dosage of other basic drugs [11,12].

2.1.2.7 Analytical method validation

The validation of the method was based on the recommendations published as Conference Reports of the Washington Conference on "Analytical methods validation: Bioavailability, Bioequivalence and Pharmacokinetic studies" [8] and of the Arlington Workshop "Bioanalytical methods validation – A revisit with a decade of progress" [9]. Recent SFSTP (Société Française des Sciences et Techniques Pharmaceutiques) recommendations were also considered [13].

Each level of the calibration curve was measured with two sets of calibrators: one set at the beginning and a second set at the end of the HPLC run. When series of patients' analyses were performed, control samples at three concentration levels (low, medium and high; i.e. 0.2, 4.5 and 9.0 µg/ml) were assayed every seven samples.

Quality control samples were used for the precision and accuracy determination. The precision was calculated as the coefficient of variation (CV %) within a single run (intra-assay) and among different assays (inter-assays). The accuracy (i.e. in fact, the inaccuracy, or bias) was the percentage of deviation between nominal and measured concentrations.

The limit of quantification (LOQ) in plasma was experimentally chosen as the minimal concentration in plasma samples which could be confidently determined in accordance with the

Arlington Workshop (recommending a deviation between measured and nominal concentrations at LOQ that do not exceed ± 20%) [9]. The limit of detection (LOD) was considered as the concentration of imatinib that provides a signal corresponding to 3 times the background signal.

2.1.2.8 Stability of imatinib

1. *Stability of plasma samples kept frozen at -20°C*: Imatinib levels of aliquoted quality control plasma samples (i.e. at 0.2, 4.5 and 9.0 μg/ml) were measured each month during 12 months. The variations of imatinib concentrations were expressed in percentages of the initial levels measured on the first day of storage.

2. *Stability of imatinib in plasma and blood samples left at room temperature (RT)*: The concentration of imatinib in QC *plasma samples* was measured immediately after preparation and after being left at RT for 24 h and 48 h.

   Anticoagulated (citrate or EDTA) *blood samples* spiked with 0.2, 4.5 and 9.0 μg/ml imatinib were left at RT. Aliquots (2 ml) were taken at 0 h, 24 h, 48 h, 72 h and 96 h, and immediately centrifuged at 1850 g for 10 min at +4°C. The plasma was collected and analyzed for imatinib, according to the above-described procedure.

3. *Stability of plasma samples after multiple freeze-thaw cycles*: Aliquots of plasma spiked with 0.2, 4.5 and 9.0 μg/ml of imatinib underwent three freeze-thaw cycles: frozen samples were allowed to thaw at ambient temperature for 2 h and were subsequently refrozen for approximately 24 h. The imatinib levels were measured in aliquots from the three consecutive freeze-thaw cycles. The variations of imatinib concentrations were expressed in percentages of the initial levels measured at the beginning of the stability study.

4. *Stability of plasma extracts into HPLC vials at RT*: Processed samples (i.e. reconstituted in 50% MeOH) containing imatinib at low, medium and high concentrations were analyzed immediately after preparation and after being left for 24 h, 48 h and 72 h at RT in the autosampler rack.

2.1.2.9 Recovery

The efficiency of the solid phase extraction was determined with control samples at 0.2, 4.5 and 9.0 μg/ml imatinib. The absolute recovery of imatinib from plasma was obtained as the peak-area response of imatinib in the processed samples. It was expressed as a percentage of the response of the same amount of imatinib (contained in the 50-μl injection volume) directly injected onto the HPLC column. The absolute recovery was similarly calculated for the IS clozapine.

2.1.2.10 Selectivity

The selectivity of our analytical method was determined by injecting onto the HPLC column 22 drugs frequently prescribed to CML- or GIST-patients at our hospital: acetylsalicylic acid (Aspirin®), diclofenac (Voltaren®), acetaminophen (Panadol®), tramadol (Tramal®), tizanidin (Sirdalud®), diltiazem (Dilzem®), verapamil (Isoptin®), atenonol (Tenormin®), torasemide (Torem®), acenocoumarol (Sintrom®), ranitidin (Zantic®), omeprazol (Antra®), metoclopramid (Primperan®), fluconazole (Diflucan®), voriconazole (Vfend®), prednisone (Prednisone®), fluoxetine (Fluctine®), citalopram (Seropram®), lorazepam (Temesta®), oxazepam (Seresta®), zolpidem (Stilnox®), allopurinol (Zyloric®).

The selectivity was also evaluated with the above-mentioned extraction procedure using blank plasma samples from six healthy subjects [8] to ascertain that no endogenous peak would interfere with the imatinib signal.

2.1.3 Results and discussion

2.1.3.1 Chromatograms

The proposed HPLC method allows the measurement of imatinib in plasma with UV detection set at 261 nm. With the gradient program used (Table 2.1), the retention times for imatinib and clozapine are 23 min and 33 min, respectively. The gradient elution program yields sharp peaks without any significant drift of the baseline.

Figure 2.1 shows the chromatogram of blank plasma, using the gradient program reported in Table 2.1. Figure 2.2 presents the chromatogram of the same blank plasma spiked with 2.5 μg/ml imatinib and 7.0 μg/ml clozapine (IS), which represents concentration commonly encountered in our patients' population. Finally, Figure 2.3 shows the chromatographic profile of another blank plasma spiked with 0.2 μg/ml imatinib and 7.0 μg/ml clozapine (IS). It is at the same scale as Figure 2.2 and corresponds to the lowest quality control concentration. Even at this low concentration, the peak has a sharp shape and is easily detectable.

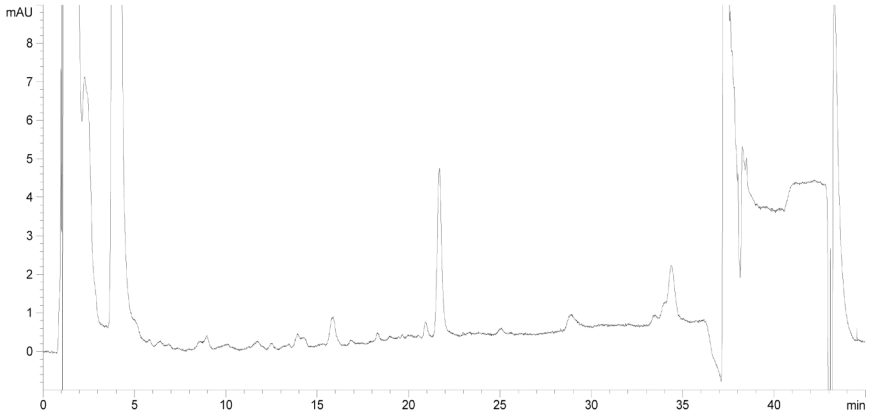

Figure 2.1. Chromatographic profile of blank plasma

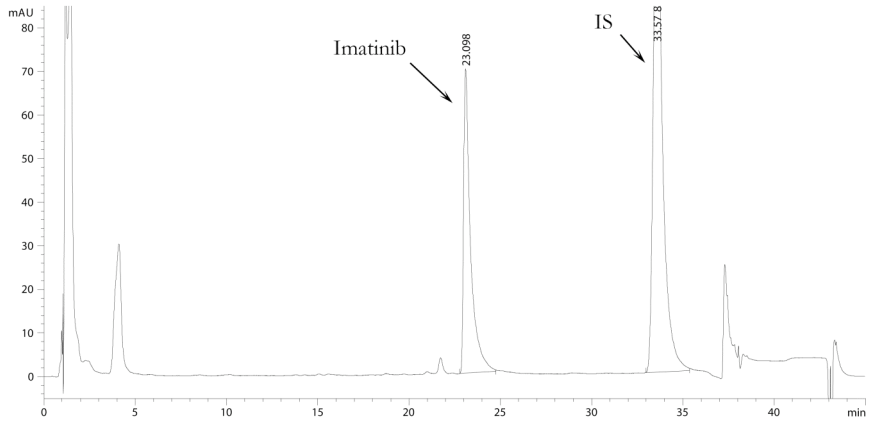

Figure 2.2. Chromatographic profile of a blank plasma spiked with 2.5 μg/ml imatinib and 7.0 μg/ml clozapine

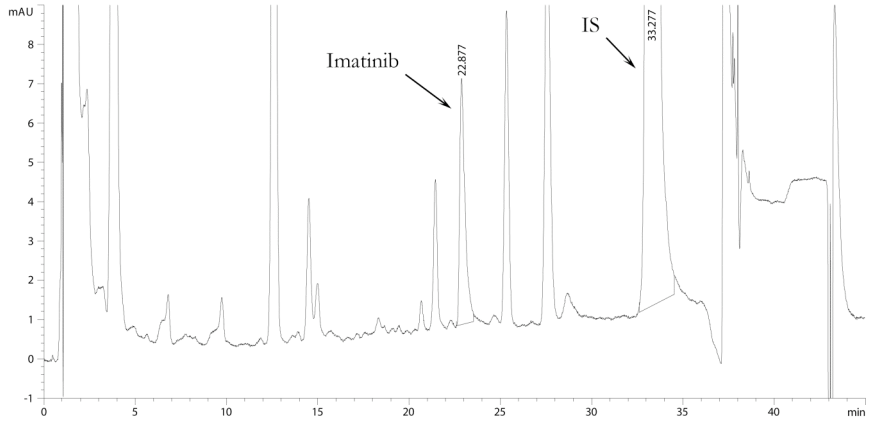

Figure 2.3. Chromatographic profile of the lowest QC plasma (0.2 μg/ml imatinib and 7.0 μg/ml clozapine)

A HPLC run of 45 min duration (including the rinsing and re-equilibration steps) can be considered lengthy. Nevertheless, it is necessary to achieve a satisfactory separation of imatinib from signals arising from endogenous plasma components and from drugs likely to be taken by this population of patients. It is noteworthy that the small peak appearing at 21.5 min is observed

in all samples (including blank MeOH/H_2O solutions subjected to SPE), and arises from the packing material smearing from the SPE cartridge during water rinsing and MeOH elution. It does not interfere with the imatinib signal at 23 min.

2.1.3.2 Mobile phase composition

Peak tailing is a common phenomenon observed with basic nitrogen-containing molecules eluted on reversed-phase columns by HPLC [14,15]. Thus, a Nucleosil 100-5 μm C18 *AB* column (i.e. a base deactivated column specifically designed for minimizing the peak tailing of base compounds [16]) was used. Furthermore, NH_4Ac was added to the mobile phase (solvent A and B) to reduce peak tailing by saturation of the stationary phase residual silanols [17].

2.1.3.3 Calibration curves

The standard curves for imatinib are satisfactorily described by 1/x weighted least-squares linear regression. The slope of the calibration curves is stable, with values averaging 1.25 (± 2.9%; $n = 6$) during the validation steps. Over the concentration range 0.1-10.0 μg/ml, the determination coefficient r^2 of the calibration curves remained always greater than 0.998.

There was originally some concern that the calibration samples prepared with citrated plasma (collected from blood from outdated transfusion bag) may not fully reflect the plasma matrix from CML and GIST patients collected on EDTA Monovettes® (polypropylene tubes known for minimal components adsorption). However, getting blood on EDTA from volunteers only for the purpose of calibration samples preparation is impracticable and ethically difficult to justify. A few reports have indeed been published, showing significant differences in concentrations measured for some drugs, related to the medium (serum *versus* plasma) or to the anticoagulant used [18-20]. For the sake of validation, the cross-validation was performed among 3 series of the three QC levels and 3 series of calibration samples (citrate *versus* EDTA). The results of the head-to-head comparison show that the anticoagulant significantly influences the imatinib results ($p < 0.05$, multi-way ANOVA). This difference was however quantitatively low and highly reproducible at every level (with a mean of 5.0% and a SD of 0.04%). Consequently, if the analysis of EDTA samples rather than of citrate samples is performed with a citrate calibration, it is recommended to correct the results accordingly. This correction has been done in our study as patients' blood samples were collected on EDTA.

2.1.3.4 Validation: precision, accuracy and LOQ/LOD

Precision and accuracy determined with the control samples are given in Table 2.2. The levels of control samples (0.2, 4.5 and 9.0 μg/ml) were selected to encompass the range of concentrations expected in the patients' plasma.

Table 2.2. Precision and accuracy of the imatinib assay in plasma

Nominal conc. (μg/ml)	Concentration found (μg/ml)		Precision CV %	Accuracy* Bias %
A. Intra-assay (*n*=6)		SD (±)		
0.200	0.199	0.003	1.3	-0.6
4.500	4.433	0.092	2.1	-1.5
9.000	9.034	0.199	2.2	0.4
B. Inter-assay (*n*=6)		SD (±)		
0.200	0.201	0.002	1.1	0.7
4.500	4.474	0.098	2.2	-0.6
9.000	9.042	0.217	2.4	0.5

*(Found—nominal)/nominal x 100.

The mean intra-assay precision is similar over the whole concentration range and always less than 2.5%. Overall, the mean inter-day precision is good with average CVs within 1.1 - 2.4%. The intra-assay deviation (bias) from the nominal concentrations of imatinib is between -1.5 and +0.4% and the range of inter-day deviation is always lower than 1.0%.

Calibration curves were established up to the highest pharmacologically relevant concentration of imatinib at 10 μg/ml, and at 0.0125, 0.025 and 0.050 μg/ml for the lowest calibration levels. This allows experimentally determining the lower limit of quantification (LLOQ) at 0.05 μg/ml. With a calibration between 0.05 μg/ml and 10.0 μg/ml, the precision (CV) of the calibration sample at 0.05 μg/ml is 6.4% and the accuracy (i.e. bias, calculated by back-calculation) at this lowest calibration level is 15.5%. Both values are thus included within the ± 20% limit recommended by the Conference Report [9]. The limit of detection (LOD) of imatinib with this assay is 0.01 μg/ml, which corresponds to a signal (0.31 mAU) equal to about three times the blank background signal at the retention time of imatinib (0.12 mAU; Figure 2.1).

2.1.3.5 Samples stability

1. *Stability at -20°C*: No evidence of imatinib decomposition was found during plasma samples storage in the freezer at -20°C for up to 1 year. After 24 months, a slight decrease can be observed as imatinib at low, medium and high quality control levels corresponds to 90.5 %, 95.4 % and 91.9 % of the respective initial values (Table 2.3). This is in fair agreement with the good stability over 9 months observed for imatinib prepared in EDTA plasma [21]. This

indicates that patients' plasma samples can be confidently stored at –20°C for a long period before HPLC analysis. In our study, the majority of samples were analyzed within 12 months after blood collection (and for a few samples within 22 months).

Table 2.3. Stability of imatinib in plasma samples kept frozen at –20°C (concentration variations expressed as % of the starting levels)

Duration (months)	Nominal concentration (µg/ml)		
	0.2	**4.5**	**9.0**
1	-0.8	-3.8	-4.0
2	-1.5	-2.2	-2.1
3	2.5	2.0	4.7
4	-1.6	-2.1	4.1
5	2.8	-2.4	1.5
6	0.4	3.6	0.9
9	0.8	0.5	3.5
12	-3.4	-2.6	-2.8
18	-1.7	-9.3	-4.5
24	-9.5	-4.6	-7.4

2. *Stability at RT in plasma and in blood samples*: The stability of *plasma samples* left at RT was ascertained up to 48 h. At the concentrations of 0.2, 4.5 and 9.0 µg/ml, the variations of imatinib levels over time were always less than ± 5% (Table 2.4) indicating that the plasma samples are stable at RT for at least 48 h.

Table 2.4. Stability of imatinib in plasma, blood and extracts samples left at RT for 24 h, 48 h, 72 h or 96 h (concentration changes expressed as % of the initial concentration; NA: not available)

Duration (h)	Medium at RT	Nominal concentration (µg/ml)		
		0.2	**4.5**	**9.0**
24	Plasma	-1.7	-4.4	-2.3
	Blood (citrated)	-1.8	-4.0	-1.2
	Blood (EDTA)	1.0	0.9	-3.5
	Extract	1.7	1.4	1.0
48	Plasma	-2.8	-4.4	-4.7
	Blood (citrated)	-1.4	-0.9	-2.7
	Blood (EDTA)	2.7	2.1	0.8
	Extract	3.9	1.8	1.1
72	Plasma	NA	NA	NA
	Blood (citrated)	-2.5	0.4	-4.0
	Blood (EDTA)	3.3	2.4	2.0
	Extract	2.9	3.1	3.4
96	Plasma	NA	NA	NA
	Blood (citrated)	-2.3	0.6	-1.9
	Blood (EDTA)	3.3	4.7	4.9
	Extract	NA	NA	NA

The stability of imatinib in *blood samples* left at RT for 96 h was checked. The variations of the levels of imatinib, at the concentrations of 0.2, 4.5 and 9.0 µg/ml respectively, was always less than ± 5% (Table 2.4) for at least 96 h, irrespectively of the anticoagulant used (citrate or

EDTA). This is important when shipment of blood samples is considered (e.g. in the case of multicentric studies).

3. *Stability after one, two and three freeze-thaw cycles*: The variations of imatinib concentrations in QC samples subjected to successive freeze-thaw cycles are reported in Table 2.5. The results indicate that no significant loss of imatinib is to be expected after up to three freeze-thaw cycles.

Table 2.5. Stability of imatinib in plasma samples after multiple freeze-thaw cycles (concentration change expressed as % of the starting concentration)

	Nominal concentration (μg/ml)		
Cycles	**0.2**	**4.5**	**9.0**
1	2.3	-2.6	-4.2
2	-0.6	-2.0	-3.3
3	0.2	-1.7	-2.8

4. *Stability of extract samples in HPLC vials at RT*: The stability of plasma extracts (i.e. reconstituted in MeOH/H_2O 50 : 50) submitted to HPLC analysis was checked at RT for 72 h and is reported in Table 2.4. The variations over time of imatinib, expressed as percentage of the initial level, were again within ± 5%. These results indicate that, taking into account the analytical variability, the processed samples are stable throughout HPLC analysis performed within up to three days.

2.1.3.6 Recovery

The mean absolute recovery of imatinib measured with the low, medium and high quality controls are 96.4 ± 2.8%, 96.6 ± 2.3% and 96.0 ± 1.9%, respectively. The clean-up procedure by SPE is reliable in eliminating interfering material from plasma, with high absolute recovery and low recovery variability.

The internal standard is fully recovered at the concentration spiked (7.0 μg/ml), and most importantly is characterized by a low variability: 101.7 ± 4.4 %.

2.1.3.7 Selectivity

At 261 nm, six different blank plasmas gave no significant interfering peaks at the retention time of imatinib. The small peak sometimes observed at about 34.5 min, i.e. near the retention time of clozapine (Figure 2.1), accounts for only a negligible part of the total area of IS (< 0.5%).

The analysis of 22 drugs prescribed for CML- and GIST-patients at our hospital confirmed the method selectivity. All drugs are eluted at retention times not interfering with imatinib determination, except for three psychotropic drugs (oxazepam, lorazepam and zolpidem;

Δ_{RT} < 0.9 min). For these three potentially interfering compounds, the entire extraction process was also conducted with plasma samples containing these drugs at their highest concentrations (C_{max}) [22]: 1.17 μg/ml, 0.04 μg/ml, 0.14 μg/ml, for oxazepam, lorazepam and zolpidem, respectively. The peak area for these compounds at their C_{max} may not be considered negligible, especially for oxazepam. However, since these drugs are mostly administered in the evening, their dosages are quite small by comparison with those of imatinib, and their concentrations presumably low at the time of sampling on the next day. Consequently, they should not interfere to any significant extent with imatinib measurements. More generally, online spectra provided by diode-array detection should be carefully examined at the time of imatinib elution (in order to exclude the presence of coeluting peaks arising from other unusual comedication taken by the patients). As far as the potentially interfering drugs are concerned, all samples from patients included in our clinical study were sent to the laboratory with the previously mentioned formulary, where all other drugs taken by patient during the latest week are indicated. The three concerned psychotropic drugs, or any unusual drug, can thus be straightforwardly identified.

2.1.4 Conclusion

This HPLC method provides a fairly robust procedure for determining imatinib in patients' plasma. Adapted from previously described more sophisticated MS-methods [21,23,24], it has been developed using instruments available in conventional hospital laboratories, requesting only a UV detector set at 261 nm. UV detection has been shown to provide the required level of sensitivity for measuring pharmacologically relevant concentrations of imatinib in patients. The increase of selectivity provided by the diode array feature is of help to exclude the possible interference of compound eluted at the retention time of imatinib or the IS. Despite the relatively prolonged time required for each analysis and the higher risk of interferences as compared to MS detection, this method provides a readily available, practicable, cheap and robust technique for hospitals. More sophisticated detection technologies such as LC-MS or MS/MS are indeed not always available for routine application in such a clinical environment. At the same time as our method validation was completed, a LC-UV procedure was also published for the analysis of imatinib and some of its impurity products in bulk drug [25], as well as in Glivec® capsules [26]. However, our method was the first to enable the measurement of imatinib by HPLC-UV using a solid phase extraction from plasma. While our publication was in press, a report just appeared also describing an assay of imatinib in human biological fluids using a valve switching technique and UV detection [27]. Since then, another LC-UV procedure for the assay of imatinib after protein precipitation as purification technique was published as a short communication. It

described a rapid and sensitive method that furthermore allowed to determine the lipophilicity of imatinib (log *P* = 1.198 at 25°C and 1.267 at 37°C) [28].

Since plasma extract samples are stable at RT in the autosampler rack, the duration of the run is not a limitation of our method and the assay can be fully automated, requiring no tedious technical supervision. In our studies, batches of samples have been analyzed over 30 h in a row without any problem. Using one single HPLC apparatus, it is possible to analyze more than 20 patient samples per run sequence. This was fairly enough for our clinical study. However, in case of the implementation of a routine dosage for imatinib –as part of a TDM program– further optimization may be probably warranted. Just recently indeed, a French group has published a description of a LC-MS/MS method that they are using for clinical monitoring of imatinib, in order to evaluate adherence to therapy, drug-drug interaction and PK-PD relationships [29].

2.2 Determination of imatinib in PBMCs by LC-MS/MS

During our study, a whole-day pharmacokinetic study was achieved with 5 consenting patients. On this occasion, peripheral blood mononuclear cells (PBMCs) were also collected from these 5 patients every 2 h for the measurement of the intracellular imatinib concentration *in vivo* by a LC-MS/MS technique. PBMCs were used as a surrogate of the cells involved in the considered pathologies (i.e. PNML, polymorphonuclear leucocytes for CML, and tumor cells for GIST).

2.2.1 LC-MS/MS: Theoretical principles

2.2.1.1 Mass spectrometry and LC-MS

Mass spectrometry (MS) is an analytical technique enabling to determine the molecular mass of ionized molecules accelerated into magnetic or electric fields. Mass spectrometers are now widely used in modern analytical laboratories, and the mass spectra they provide enable analysts to determine the molecular masses. Additionnaly, when using high resolution technology, structural information of compounds in complex samples can be obtained [1,2].

Being able to couple MS to LC was a technological challenge because of the critical step of eliminating the aqueous mobile phase during the ionization process. Among the possible interfaces and ionization sources that have been developed for that purpose, the electrospray interface has been chosen for the present study. The application of a high electrical potential at the surface of the liquid flowing through the extremity of a capillary is necessary for the

production of charged droplets. They disintegrate upon drying by an intense heat source to ultimately generate ions that are accelerated into the detector [1,2]. The produced ions are then guided by a quadripole detector, onto which an electrodynamic field of a given radiofrequency is applied for the selection of ions with specific m/z ratio.

2.2.1.2 Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) implies the selection by the first analyzer (i.e. quadripole in our case) of a parent ion (generally the molecular ion), which is directed by magnetic fields into the so-called collision cell (ie. in fact a second quadripole). This cell contains a neutral gas (argon) into which the ion disintegrates to produce a number of fragments (daughter or product ions). One product ion of enough intensity is then selected by a second analyzer (third quadripole) before its detection by a photomultiplier [1].

2.2.2 Materials and methods

2.2.2.1 PBMC isolation from blood

The PBMCs isolation procedure is based on the method previously developed in our laboratory for the intracellular measurement of the anti-HIV drugs by Colombo *et al* [30]. On the study day, samples were immediately taken (within 10 min) to the laboratory and processed. PBMCs were isolated using Vacutainer® CPT (Cell Preparation Tubes, Becton Dickinson, Allschwil, Switzerland), according to the manufacturer's recommended procedure [31]. These tubes contain citrate as anticoagulant, a polyester gel layer and a Ficoll[1] solution enabling the direct separation of mononuclear cells from other blood components. One tube per patient (approximately 8 ml of blood) was collected, gently inverted 8-10 times for careful homogenization with the anticoagulant, and centrifuged within 5 min (1650 g for 20 min at RT without brake; Centrifuge J-6B, Beckman, Fullerton, USA). The thin mononuclear cell layer was carefully mixed with plasma and transferred into a polypropylene tube (15 ml) pre-chilled at 4°C on an ice bath. The entire cell washing procedure was performed using pre-chilled PBS (phosphate-buffered saline) kept at +4°C. The centrifuge (Benchtop Universal 16R, Hettich, Bäch, Switzerland) was also maintained at +4°C to inhibit enzymatic activity and to prevent active drug efflux out of cells.

The collected cells suspended into the plasma were first centrifuged at 4°C for 10 min, and after discarding the supernatant, they were washed with 10 ml cold PBS solution containing 2%

[1] Ficoll is a neutral highly branched, high-mass, hydrophilic polysaccharide.

FBS (fetal bovine serum). After careful cell homogenization, the tube was centrifuged for 10 min at 650 g (+4°C) and the supernatant discarded. The washing procedure was repeated twice. The third (final) supernatant wash solution was completely aspirated off and an exact volume of 1.0 ml of PBS was added to the cell pellet. Cells were carefully homogenized and suspended, and a 20-µl aliquot was taken out from them and diluted 1 : 10 with 180 µl PBS in a 0.5-ml Eppendorf tube. This aliquot was homogenized for cell counting within 10 min, allowing the total number of PBMC contained in each patient's pellet sample to be calculated, and used for normalizing the intracellular drug concentration determined by LC-MS/MS. The Laboratory of Hematology of our hospital performed the cell counting with a Coulter counter.

The remaining 980-µl cell suspension was centrifuged at 650 g for 10 min at 4°C. The supernatant was aspirated off, leaving the PBMCs pellet at the bottom of the vial which was immediately stored at –20°C up to the day of the analysis.

2.2.2.2 Imatinib measurement in PBMCs pellets

The development of the LC-MS/MS methodology used for quantifying imatinib levels in PBMCs was carried out by an assistant-pharmacist[2] during her Pharmacy diploma [2]. It was based on various publications of LC-MS measurement of imatinib in plasma [21,24,32], as well as on publications on the intracellular quantification of cytotoxic [33] and anti-HIV drugs [30].

It is also worth noting that this LC-MS/MS analysis has been applied for the quantification of imatinib levels in liver biopsies. This measurement was carried out as part of a collaborative work with the Institute of Clinical Pharmacology of the University of Bern, in a study aiming at assessing the antifibrotic effect of imatinib in rat livers. This study was not directly related to the present thesis work, and is therefore not presented here, but can be found in an article recently published in the *Journal of Hepatology* [34]. Moreover, such a technique was also used to measure imatinib inside cultured cells that have been knocked down for P-gp by an Austrian research group (see § 6.2 for details).

The analysis of PBMCs samples collected during our clinical study was performed by the Principal Laboratory technician[3] in charge of the LC-MS/MS instrument of the Division of Clinical Pharmacology.

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[2] Aurélie Fayet
[3] Alexandre Béguin

1. *PBMCs extraction procedure*: 100-µl PBS buffer was added to the PBMCs pellets, as well as 100 µl acetonitrile (ACN, containing 0.05 µg/ml clozapine as IS) in order to denature and inactivate proteins and enzymes. The resulting suspensions were vortexed to ensure adequate mixing and were subsequently incubated at 37°C for 1 h. The mix was finally sonicated for 5 min in an ultrasound bath for cell lysis and centrifuged for 10 min at 20°C and 20'000 g (Benchtop Universal 16R centrifuge, Hettich, Bäch, Switzerland).
   100 µl of supernatants diluted with 100 µl mobile phase A (NH_4Ac buffer 10 mM + 1% acetic acid) were introduced into 200-µl HPLC microvials (Agilent, Böblingen, Germany) and a volume of 10 µl was used for LC-MS/MS analysis.

2. *LC-MS/MS quantification*: The LC-MS/MS system consisted of a TSQ Quantum Discovery tandem triple-stage quadripole (Thermo Finnigan, San Jose, USA), coupled with an Agilent 1100 liquid chromatography system (Agilent, Böblingen, Germany) equipped with a binary pump, an autoinjector and an on-line degaser system. The temperature of the autoinjector was set at 20°C during the operation. HPLC vials were maintained at 20°C in the autosampler. Chromatographic separations were performed on a Symmetry Shield RP8 3.5 µm, 30 mm length x 2.1 mm id column (Waters, Milford, USA), thermostated at 30°C. The binary solvent system was NH_4Ac buffer 10 mM + 1% acetic acid (A), and MeOH + 1% acetic acid (B) used in gradient mode and followed by a rinsing and re-equilibration program for up to 16 min. The LC-elute was introduced into the electrospray source without splitting. An adequate MS/MS tune settings for imatinib/metabolites/clozapine (IS) was chosen [2]. A desolvation temperature of 350°C, a spray voltage of 4000 V, a source CID (collision-induced dissociation) of 10 V, a sheath gas flow rate of 40 units, an auxiliary gas flow rate of 10 units and a collision gas (argon) of 1.0 mTorr were used. Multiple reactions monitoring (MRM) was selected in the positive ions mode for all analytes.
   It is noteworthy that this LC-MS/MS method allows the intracellular measurement of imatinib, its main metabolite N-desmethylimatinib (CGP74588) and of some oxygenated metabolites of imatinib (as yet unidentified).

3. *Imatinib levels calculation*: After PBMC extracts quantification, imatinib levels in PBMCs may be expressed either in ng per 10^6 cells, taking into account the number of cells determined in the sample at Hematology Laboratory, or in ng/ml ("intracellular" concentration), assuming a 0.4 pl PBMC volume [35].

The limit of quantification of the assay was 10 μg/l. Mean intra-day and inter-day precision for intra-PBMC imatinib was 2.9 and 6.3%, respectively. Finally, the mean intra-day and inter-day accuracy was 1.9 and 5.9%, respectively.

2.3 *MDR1* genotyping

Since the *MDR1* 3435C>T polymorphism is known to influence P-gp expression *in vivo*, with potential consequence on the disposition of its substrates [36-38], and since P-gp markedly affects the imatinib intracellular concentration (see Chapter 6), the genotyping of the *MDR1* gene was considered as a biological covariate in the present clinical study. The 3435C>T polymorphism was therefore assessed in consenting patients using a restriction length polymorphism method previously established at the Clinical Chemistry Laboratory of our hospital[4].

2.3.1 Theoretical introduction

Examination of the exon 26 of *MDR1* gene reveals that the SNP 3435C>T is located at position 176 [39], with the 3435T variant resulting in the disappearance of a restriction site (GATC between positions 172 and 176) recognized by three restriction enzymes (DpnII, MboI and Sau3AI).

To assess the genotype of a patient, the procedure implies the extraction of DNA from the buffy coat of patients' blood samples and subsequent purification using a suitable solid-phase extraction kit for DNA. A sequence of *MDR1* exon 26 contained in this DNA is then amplified using a polymerase chain reaction (PCR)[5] method, before being restricted by the MboI enzyme (i.e. cutting amplicons if the restriction site is recognized). In our case, the expected restriction enzyme-digested fragments are 143- and 46-bp for the CC genotype at nucleotide 3435; 189-, 143- and 46-bp for CT; and 189 bp for TT. Finally, the fragments are visualized as bands on an agarose gel after electrophoresis and subsequent ethidium bromide staining.

[4] References: "Genomic DNA preparation" and "Restriction fragment length polymorphism MDR1 C3435T" SOPs issued by the Clinical Chemistry Laboratory at CHUV.
[5] As a reminder: PCR is a process in which a particular DNA fragment from a mixture of DNA chains is rapidly replicated, producing a large, readily analyzed sample.

2.3.2 Materials and methods

2.3.2.1 DNA purification from pellet samples

Purification of patients' genomic DNA was carried out using QIAamp® DNA kits (Qiagen, Basel, Switzerland). A 200-µl aliquot of solid cellular pellet samples (obtained after plasma collection and containing buffy coat) were added to Eppendorf tubes containing 20 µl Proteinase K, prior to the addition of 200 µl alkaline lysis buffer (AL buffer, containing a chaotropic salt). After mixing by pulse-vortexing for 15 s, the tubes were incubated at 56°C for 10 min and briefly centrifuged at 13'000 g (Genofuge® 16M, Techne, Cambridge, UK) to remove any drop present on the inside of the lid. A 200-µl volume EtOH was added and the mixes were vortexed again for 15 s and briefly centrifuged.

The mixtures were then carefully applied to SPE columns specifically designed for DNA extraction (QIAamp Spin columns), placed into 2-ml collection tubes, and centrifuged at 10'000 g for 1 min. The columns were then put into clean 2-ml collection tubes while the previous tubes containing the filtrate were discarded. 500 µl of a first wash buffer (also containing a chaotropic salt with large amount of EtOH) was added, and the same procedure was repeated (centrifugation and discarding of collection tubes). 500 µl of a second wash buffer (containing EtOH) were subsequently added, followed by a centrifugation at 13'000 g for 1 min. The collection tubes were finally replaced by 1.5-ml Eppendorf tubes (without lids) before the addition of 200 µl water in the columns and incubation for 5 min. The columns were centrifuged at 10'000 g for 1 min to eluate DNA out of them. DNA concentration in Eppendorf tubes was finally checked by photometry (260 nm).

2.3.2.2 PCR mix preparation

The PCR mix was prepared in a specifically assigned protection hood (for preventing cross-contamination). Various reagents were mixed in the following order: 5.0 µl incubation buffer 15 mM $MgCl_2$ x 10, 1.0 µl nucleotides 10 mM (dNTPs from Q-Biogene, Basel, Switzerland), 2.0 µl primers (F2 and R2 M29445), 0.5 µl Taq DNA polymerase 5 U/µl from Q-Biogene (all quantities for one sample). The mix was vortexed and 45-µl aliquots were distributed in PCR tubes. The primers' sequences were as followed: forward (F2) 5'-TGGCAAAGAAATAAA GCGAC-3'; and reverse (R2) 5'-GACTCGATGAAGGCATGTA-3'.

Outside of the hood, 5 µl of patients' genomic DNA were added to each PCR tubes, together with two drops of paraffin. The mixtures were finally vortexed and briefly centrifuged at 13'000 g.

2.3.2.3 PCR amplification

PCR amplification was done with a Touchgene gradient thermal cycler (Techne, Cambridge, UK) with the program described in Table 2.6. The above-mentioned primers (F2 and R2) are shown on Figure 2.4.

Table 2.6. Touchgene PCR program

Phases	Cycles #	T [°C]	Duration	Purpose
1	1	94	2 min	Denaturation
2	10	94	15 s	Denaturation
		58	30 s	Hybridation
		72	40 s	Elongation
3	25	94	15 s	Denaturation
		58	30 s	Hybridation
		72	1st cycle: + 20 s 25th cycle: + 6 min	Elongation (for increasing amount of materials)
4	1	72	7 min	End of elongation
5	1	4	∞	Cooling

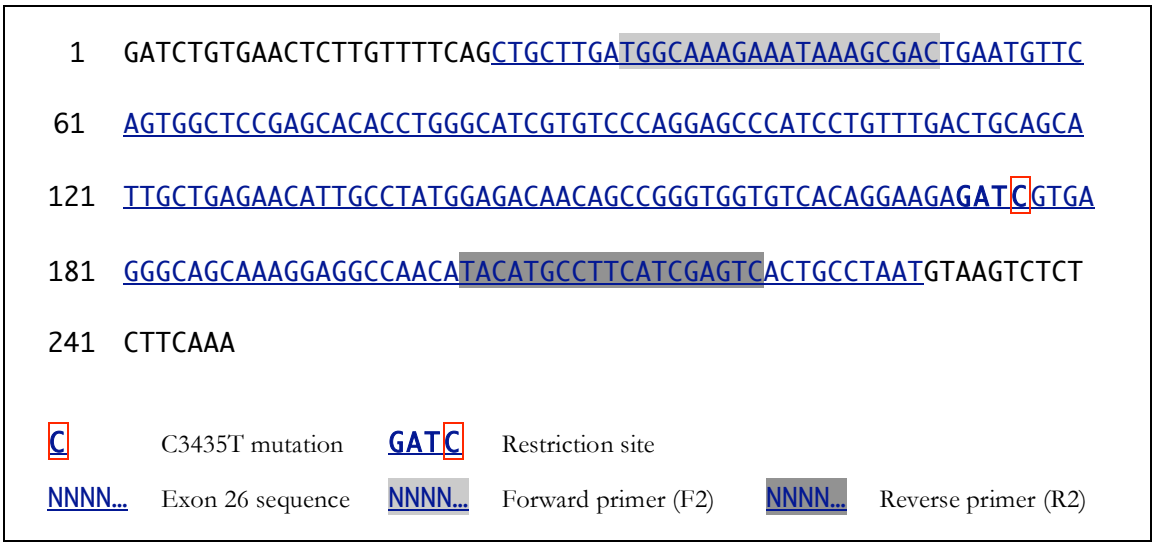

Figure 2.4. GenBank sequence # M29445

2.3.2.4 Restriction

10-µl aliquots of the 189 bp PCR product (obtained after amplification) were pipeted into 500-µl Eppendorf tubes before the addition of 1 µl MboI enzyme and one drop of paraffin. The mix was vortexed, briefly centrifuged and incubated overnight at 37°C. In the absence of mutation, MboI was able to cut the amplified sequence at the restriction site (Figure 2.4).

2.3.2.5 Agarose gel electrophoresis

The DNA fragments produced during the above-mentioned experiments were finally separated by electrophoresis with a high-resolution agarose gels consisting of 3% MetaPhor® in TBE buffer (Tris/borate EDTA, pH 8.0). MetaPhor® (Cambrex,, Verviers, Belgium) is an intermediate melting temperature agarose. Three gels were prepared by microwave dissolution according to the manufacturer's recommended procedure.

Before migration, 2 µl DNA loading buffer 6x were added to the restriction mixes. Each sample was charged onto the gels, as well as a DNA molecular mass marker from the laboratory (no. VIII). A CC control sample and a blank control (water) were also charged. The migration was carried out for 2 h under a 75-V voltage.

After migration, the gels were incubated with ethidium bromide 0.5 µg/ml for 30 min and washed with distilled water. They were then scanned with a fluorimetry densitometer Typhoon (Amersham Biosciences, Piscataway, USA) and the fragments were identified by comparison with the DNA molecular mass markers.

2.4 Urinary 6β-OH-cortisol/cortisol measurement by LC-MS

2.4.1 Background

As previously stated (see § 1.5.1 and 1.8.1), the 6β-hydroxycortisol/cortisol ratio, used as a putative index of CYP3A4 activity (one of the biological covariate of our study), was determined in urine spots collected from patients simultaneously to the blood sample. A laboratory technician[6] at PCL has carried out the analysis using a method previously developed by an assistant-pharmacist[7] during his Pharmacy diploma [1]. It is noteworthy that an HPLC-UV method had previously been evaluated in our laboratory [40], but this technique lacked the required level of sensitivity and specificity, because both compounds are frequently present at very low levels in the collected urine. The liquid intake and time of urine collection was not controlled in the study and the concentrations of 6β-hydroxycortisol and cortisol in urine collection were thus highly variable. The development of the present LC-MS/MS method was therefore required for the measurement of endogenous cortisol and 6β-hydroxycortisol measurements in patients' urine. Theoretical considerations on such a technique have already been exposed.

[6] Alexandre Béguin
[7] Hugo Figueiredo

2.4.2 Materials and methods

2.4.2.1 Extraction from urinary samples

Urinary samples were purified using a solid-liquid SPE technique with Chromabond® XTR (Macherey-Nagel, Düren, Germany) based on a publication by Ohno *et al* [41]. A 3-ml urine aliquot was centrifuged for 5 min at 1800 g (Centrifuge J-6B, Beckman, Fullerton, USA) to eliminate the precipitate, which can appear over time during the storage at -20°C. A 1-ml aliquot of the clear supernatant was taken and vortexed in an Eppendorf vial with 100 µl of a solution of cortisol-d4 (IS) at 250 ng/ml. Finally, 1 ml of this solution was directly loaded on the Chromabond® XTR cartridge, which does not require any pre-conditioning step.

The sample was absorbed into the solid phase and maintained for 10 min onto the cartridge, before elution with 2 x 4 ml of ethyl acetate (AcOEt). This eluted AcOEt phase was evaporated to dryness under a nitrogen stream. The solid residue was reconstituted with 200 µl of MeOH/H_2O 50 : 50 (v/v). A 50-µl aliquot of this reconstituted sample was diluted with 75 µl of water to attain the desired MeOH/H_2O 20 : 80 (v/v) final solvent composition (similar to the solvent composition at the beginning of the gradient program). 20 µl of this last solution was injected on the LC-MS/MS system.

2.4.2.2 LC-MS/MS quantification

The chromatographic method used for the separation of cortisol and 6β-hydroxycortisol is an adaptation of various previously published methods [41,42]. The same instrument as for the intracellular measurement of imatinib was used (TSQ Quantum Discovery tandem triple-stage quadripole; Thermo Finnigan, San Jose, USA), coupled to an Agilent 1100 liquid chromatography system (Agilent, Böblingen, Germany). The mobile phase consisted of 0.1% formic acid (A) and MeOH + 0.1% formic acid (B). The chromatographic column was Nucleosil 100-3 µm C8 *HD* (Macherey-Nagel, Düren, Germany). A stepwise gradient was used at a typical flow rate of 0.3 ml/min. These conditions provided a good separation of the two compounds (6β-hydroxycortisol eluted in 3.2 min, and cortisol and IS in 9.0 min). An adequate MS/MS tune settings for cortisol/6β-hydroxycortisol/cortisol-d4 (IS) was chosen [1]. A desolvation temperature of 350°C, a spray voltage of 4500 V, a source CID (collision-induced dissociation) of 10 V (6β-OH-cortisol) and 15 V (cortisol and IS), a sheath gas flow rate of 60 units, auxiliary gas flow rate of 5 units and a collision gas (argon) of 1.0 mTorr were used.

The limits of quantification of the assays were 1 μg/l for cortisol and 10 μg/l for 6β-hydroxycortisol. Mean inter-day precision and accuracy were 7.4 and 7.2%, and 9.1 and 3.7% for cortisol and 6β-hydroxycortisol, respectively.

2.5 Plasma α_1-acid glycoprotein measurement

2.5.1 Theoretical introduction

The third main biological covariate of our PK analysis (AGP plasma level) was measured at the Laboratory of Clinical Biochemistry and Psychopharmacology Unit in Cery. The principle of the method implies the precipitation of AGP with a specific rabbit antiserum that can then be analyzed by turbidimetry [43].

Turbidimetry is an analytical method based on a phenomenon whereby the intensity of light, passing through a medium with dispersed particles, is attenuated by scattering. It is worth noting that, in turbidimetry, the intensity of transmitted light (i.e. unscattered) is measured, whereas in nephelometry the scattered light is measured.

The limit of quantification of the assay was 0.16 g/l. Mean intra-day and inter-day precision were 1.9 and 2.4%, respectively, for a 0.62 g/l concentration, and 1.0 and 1.5%, respectively, for a 2.22 g/l concentration.

2.5.2 Materials and methods

The automate instrument used was a COBAS INTEGRA 400 system (Roche Diagnostics, Basel, Switzerland). A 100-μl aliquot of patients' plasma samples was pipeted into suitable tubes in our laboratory. After reception at Cery and dilution, polyethyleneglycol (used as reaction accelerator) and rabbit antiserum T were added. The rabbit antiserum precipitated AGP, which could then be quantified by turbidimetry at 340 nm, using a calibration method (with Serumproteins T standards). The results are given in g/l.

2.6 References

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Chapter 3 Population pharmacokinetic study

This chapter will first provide a detailed description of the overall procedure used for clinical data collection from patient medical files and their gathering into a suitable database. Their integration into the population pharmacokinetic modeling performed with the NONMEM® software will then be largely presented.

The results of a subsequent pharmacokinetic-pharmacodynamic (PK-PD) analysis will be exposed in a specific chapter (i.e. Chapter 4).

3.1 Theoretical introduction

3.1.1 Imatinib pharmacokinetics

Our current knowledge of imatinib pharmacokinetics previously given in § 1.3.4 has been gathered during the phases I, II and III of the clinical development of imatinib, which have been recently published [1-6]. Among the two stringent studies applying a population approach [2,4], one of them [4] has indicated that imatinib clearance tends to increase upon Glivec® treatment in GIST patients, followed over 12 months. Accordingly, imatinib levels in patients whose dosage was increased (upon progression) were substantially lower than those at the beginning of the treatment. By contrast, a 25% decrease in imatinib overall clearance was reported in another study with CML patients [2], albeit followed up for only one month. As recently stated by Leveque *et al*, the clinical relevance of imatinib PK variability deserves investigation, as well as the identification of individual kinetic determinants that could modulate the clinical response [7]. Weight, creatininemia, albuminemia, hemoglobinemia and some other covariates have already been assessed [2,4]. To our knowledge however, no formal study assessed the effects of the three important covariates mentioned above –namely *MDR1* expression, CYP3A4 activity and AGP plasma levels– on imatinib PK parameters. Only one study, published in an abstract form, has correlated *MDR1* single nucleotide polymorphism (SNP) and CYP3A4 activity to imatinib area under the curve (AUC) [8]. Finally, no data are available about the intracellular levels of imatinib into patients' cells, and a single study only had assessed *in vitro* the intra/extracellular ratio of this drug in human plasma [3].

As previously stated (§ 1.8), the major goals of our clinical pharmacokinetic study were thus (1) to characterize the population PK profile of imatinib in CML and GIST patients, (2) to

evaluate the influence of various demographic and biological covariates on imatinib absorption and disposition, (3) to assess the specific role of AGP on imatinib PK, and (4) to further explore the intracellular uptake of imatinib *in vivo*.

3.1.2 The NONMEM approach

As mentioned in § 1.6, population pharmacokinetics aims at describing the pharmacokinetic variability in terms of a series of interrelated factors [9]. They can be evaluated, for instance, using the NONMEM approach.

3.1.2.1 General principles

NONMEM stands for "NONlinear Mixed Effects Model". It is a computer program, written in FORTRAN[1] 77, and designed to fit general statistical (nonlinear) regression-type models to data. This program was developed by the NONMEM Project Group at the University of California at San Francisco, a group of researchers in the Division of Clinical Pharmacology. The first version appeared at the beginning of the 1980s. It was able to analyze data described by a regression model with both fixed and random effects, and was thus notably developed for analyzing population pharmacokinetic data [10,11]. NONMEM® is now the most widely used software for this type of analysis and has been employed for numerous drugs, such as phenytoin (yielding the well-known "orbit plot" [12]) and angiotensin antagonists (e.g. in a study realized at the Division of Clinical Pharmacology at Lausanne PCL Division [13]).

This regression software describes the observed concentration-time data in terms of deterministic and probabilistic parts [9,13]:

1. *Deterministic part*: Several fixed effects parameters θ, which may include the mean values of the pharmacokinetic model parameters ϕ_j or parameters relating the model to the demographical, physiological or external variables described above.
2. *Probabilistic part*: Two types of random effect parameters characterizing the variability around the fixed effects:
   • ω^2: variance of θ, i.e. the interindividual variability. Thus, an individual has his own parameter value $\theta_j = \theta + \eta_j$, where η_j follows a centered normal distribution with a ω^2 variance.

[1] Fortran (also FORTRAN; FORmula TRANslator) is a computer programming language originally developed by IBM in the 1950s and still used for scientific computing and numerical computation. It is sometimes considered as the earliest "cross platform" programming language.

• σ^2: residual intraindividual variability due to random fluctuations in an individual's parameter values, measurement errors and all sources of errors not accounted for by the other parameters. An i^{th} observation in the j^{th} individual (y_{ij}) thus differs from the corresponding prediction $\hat{y}$ by a residual error of ε_{ij} (following a centered normal distribution with a σ^2 variance).

The function of NONMEM is thus to estimate the average values of these three parameters.

3.1.2.2 The general model

A general model in NONMEM can be written in the following generic form [13]:

$$y_{ij} = f(x_{ij}, \phi_j) + \varepsilon_{ij} \qquad \text{where} \quad \phi_j = g(z_j, \theta) + \eta_j \tag{1}$$

As previously stated, y_{ij} is the i^{th} observation (typically the plasma concentration) from the j^{th} individual, f is a specified pharmacokinetic (or even pharmacodynamic) model, which is function of known quantities x_{ij} (e.g. dose, time) and parameters ϕ_j (e.g. clearance, distribution volume). The parameters ϕ_j are functions of fixed effects z_j (e.g. age, height) and fixed effects parameters θ previously cited. As already mentioned, ε_{ij} corresponds to the residual error and η_j to the interindividual error.

By using NONMEM, the aim is to determine the best model to fit the experimental data, in order to obtain estimates of the average values of θ, ω^2 and σ^2. The fitting method used by NONMEM is the one based on the extended least squares criterion [14]. The model has to be selected among classical PK models such as one compartment, two compartments, linear or nonlinear kinetics for PK variables (e.g. concentration), or among PD models such as E_{max} for effect variables.

3.1.2.3 NM-TRAN and PREDPP

Two additional components are distributed with NONMEM: NM-TRAN and PREDPP. The first one stands for "NONMEM translator" and is a language processor and data preprocessor that translates commands and data files in NONMEM input files. The second one stands for "Prediction for Population Pharmacokinetics". Whereas NONMEM is a general nonlinear regression tool, PREDPP is specific to the types of predictions that arise in pharmacokinetic models. It is an extensive library of Fortran subroutines, corresponding to various predefined models (e.g. ADVAN and TRANS subroutines) [13,14].

3.2 Materials and methods

3.2.1 General study procedure

These paragraphs summarize the main logistical and practical aspects of the study protocol and its amendment presented in Chapter 1 (and which can also be found in Appendices 1.1 to 1.5). The study was approved in 2002 by the Ethics Committee of the Lausanne Faculty of Medicine (see Appendices 1.3 and 1.5).

At the occasion of a scheduled medical visit for the usual follow-up of their conditions, the patients were given oral and written informations on the objectives and on the logistical details of the study (see "Feuille d'information aux patients" in Appendix 1.2). After obtaining the written informed consent, a 5-ml blood sample was drawn together with the standard routine sampling (chemistry, hematology). For practical reasons, one blood sample was taken per visit, if possible during the imatinib elimination phase (i.e. at least 4 h after the latest dose intake). It had been anticipated that each patient would provide at least two samples at 3-month intervals (to assess intra- and interindividual variability). Moreover, as previously mentioned, plasma and intracellular (i.e. intra-PBMC) imatinib pharmacokinetics was also studied for a small number of consenting patients (i.e. 5 subjects) over a whole dosing interval in the Clinical Research Unit at the Division of Clinical Pharmacology. This ancillary study has required an amendment of our protocol (Appendices 1.4 and 1.5).

Clinicians had to report on a specific Case Report Form (CRF, see Appendix 3.1) the exact time of blood sampling, the daily regimen, as well as the dose, the date and the exact time of the last intake of Glivec®. Comedication taken by the patient during the last week and relevant information such as side effects and clinical evolution (at the time of blood collection and during the preceding month) also had to be reported in details. Cancer progression markers were considered (hematological, cytogenetic and molecular response for CML, and tumor evolution criteria (RECIST) [15] for GIST). All these data were reported at each routine medical visit, and were checked and validated, at the end of the study, by direct access to the medical files of each patient. Laboratory test results (such as creatininemia) were obtained from the central computer system of our hospital (Molis®), gathering all routine laboratory results at CHUV. All data have been introduced in an Access® database (version 2000, Microsoft Co., Redmond, USA), designed in collaboration with the PCL Data manager[2]. For the whole-day study, a special CRF (Appendix 3.2) was elaborated. It was directly filled at PCL Clinical Research Unit.

[2] Ali Maghraoui

In addition, patients were asked to provide a spot of urine sample (ca 10 ml), for the determination of the 6-β-hydroxycortisol/cortisol ratio. These blood and urine samples were taken at each of the patients' routine medical visit. Blood samples (5 ml) were collected in Monovettes® (Sarstedt, Nümbrecht, Germany) with K-EDTA as anticoagulant and brought or sent together with urine samples (Monovettes®) to the Laboratory of Clinical Pharmacology.

3.2.1.1 Sample storage

Blood samples were immediately centrifuged (1800 g, +4°C, 10 min) at the laboratory (Centrifuge J-6B, Beckman, Fullerton, USA). The plasma was separated and transferred into polypropylene test tubes before storage, together with urinary sample at -20°C, prior to LC-UV analysis. During the whole-day pharmacokinetic study, PBMC samples were immediately processed after collection and cells pellet were stored at -20°C before LC-MS/MS analysis (§ 2.2).

The cellular (erythrocytes and buffy coat) bottom layer obtained after blood centrifugation has been kept for subsequent PCR analysis of P-glycoprotein (P-gp) polymorphism, relevant cytochromes P450 polymorphism analysis, or for the analysis of *BCR-ABL* and *KIT* expression and mutation in patients.

DNA samples have been coded to preserve patients' anonymity. The remaining samples or the non-analyzed samples will be eliminated one year after the end of the study, but in January 2007 at the latest.

3.2.1.2 Samples analysis

Imatinib in plasma was analyzed on a semi-routine basis (batch series of approximately 20 patients' samples) by the LC-UV method previously mentioned [16]. 6β-hydroxycortisol and cortisol in urinary samples were quantified by LC-MS/MS at the Quantitative Mass Spectrometry Facility (qMSF) of CHUV (method reported in § 2.4). Plasma AGP levels were measured by the Laboratory of Clinical Biochemistry and Psychopharmacology Unit in Cery (see § 2.5).

3.2.2 Population pharmacokinetic analysis with NONMEM®

Population PK analysis was carried out with the previously described NONMEM® program. The description of the analysis presented here corresponds to the article that has just been accepted for publication by an international journal [17].

Data from 59 patients, providing a total of 321 plasma samples, were collected during 3 years for the population PK analysis. These patients included 38 with GIST, 20 with CML and 1 with ALL, who received imatinib at various dosage regimens (daily doses ranging from 150 to

800 mg). All patients were pooled in our analysis, regardless of their medical history. Their median age was 55 years (range 20-79), their median body weight was 71 kg (44-110), and their median height was 172 cm (152-189); 26 patients were female (Table 3.1). Most peripheral blood samples were drawn periodically at 1- to 6-month intervals on follow-up visits, along with routine laboratory tests. The median number of measurements for each patient was 4 (range 1-14). All samples were obtained under steady state conditions (i.e. unchanged dosage for at least one month). Additional measurements were taken in 5 consenting patients over one dosing interval to obtain a detailed concentration-time profile, with 9 peripheral blood samples drawn between 0 and 8 h after drug intake.

Table 3.1. Characteristics of the 59 patients (corresponding to 321 samples) evaluated in the population pharmacokinetics analysis of imatinib

Characteristic	Patients
Pathology diagnosis (No.)	
GIST	38
CML	20
ALL	1
Gender (No.)	
Men	33
Women	26
Age (y)	
Median	55
Range	20-79
Body weight (kg)	
Median	71
Range	44-110
Height (cm)	
Median	172
Range	152-189
AGP plasma levels (g/l)	
Median	0.9
Range	0.4-3.2
MDR1 genotype (No.)	
3435CC	5
3435CT	22
3435TT	9
CYP3A4 ratio (UCR)	
Median	3.6
Range	0.7-38.5
CYP3A4 inducers	
Carbamazepine, rifampicine, dexamethasone	3
CYP3A4 inhibitors	
Verapamil, diltiazem, fluoxetine, fluvoxamine, amiodarone, éthinylestradiol, fluconazole, voriconazole	9

UCR = urinary cortisol ratio

During this same single-day study, intracellular concentrations of imatinib were also measured in peripheral blood mononuclear cells (PBMC; used as a surrogate of target cells) from four peripheral blood samples. These samples were drawn before and 2, 4 and 6 h after drug intake using Vacutainer® CPT (Cell Preparation Tubes, Becton Dickinson, Allschwil, Switzerland),

according to the manufacturer's recommended procedure and to the method previously developed in our laboratory for the intracellular measurement of anti-HIV drugs [18]. All tubes were processed at +4°C and the collected cells were washed three times with cold PBS, prior to cell counting with a Coulter counter and imatinib measurement using the LC-MS/MS method previously described (see § 2.2.2).

In addition to accurate dosing and sampling time information, the following data were recorded for each patient: body weight, gender, age, height, creatininemia (CRT, in μmol/l) and concomitant medication intake (Table 3.1).

Furthermore, the genotype of *MDR1* was determined in 36 patients for the 3435C>T single nucleotide polymorphism (SNP), known to be associated with P-gp expression level [19]. The evaluation of the CYP3A4 function was achieved by measuring the 6β-hydroxycortisol/cortisol ratio in a 10-ml spot of urine collected at the same time as 164 peripheral blood samples [20]. These urine samples were stored at -20°C until analysis (see below). As previously stated, the measurement of endogenous levels of 6β-hydroxycortisol/cortisol in urine represents an attractive approach to assess the CYP3A4 activity, as it does not require the administration of any exogenous probe substance [20]. Finally, AGP plasma levels were assessed in 278 plasma samples. The influence of *MDR1* genotype, CYP3A4 activity and AGP plasma levels on imatinib PK was thus evaluated in the patient subsets.

3.2.3 Pharmacokinetic modeling

3.2.3.1 Demographic covariates analysis

A one-compartment and a two-compartments model with first-order absorption were tested. The first one is described by the following differential equations:

$$\frac{dA_1}{dt} = -k_a \cdot A_1 \quad (2)$$

$$\frac{dA_2}{dt} = k_a \cdot A_1 - CL \cdot C_{tot} \quad (3)$$

where A_1 and A_2 are the amounts of imatinib in the absorption and central compartments, k_a the first order absorption rate, C_{tot} the measured drug concentration (corresponding to the A_2/Vd ratio, and where Vd is the volume of distribution) and CL the clearance.

Three demographic covariates (body weight, gender and age), as well as the pathology diagnosis, were then sequentially incorporated in the model and tested for significance, leading to the following equations (final expressions):

$$CL = \theta_a + \theta_1 \cdot \frac{(BW - BW_{mean})}{BW_{mean}} + \theta_2 \cdot q - \theta_2 \cdot (1-q) + \theta_3 \cdot \frac{(AGE - AGE_{mean})}{AGE_{mean}} + \theta_4 \cdot p - \theta_4 \cdot (1-p) \quad (4)$$

$$Vd = \theta_b + \theta_5 \cdot q - \theta_5 \cdot (1-q) \quad (5)$$

where body weight (BW) and age (AGE) are expressed as the relative deviation of the individual BW and AGE from the population mean (BW_{mean} = 70 kg and AGE_{mean} = 50 years, respectively). Dichotomous variable were used for gender (q = 0 for female and 1 for male) and for the pathology diagnosis (p = 0 for CML and 1 for GIST).

3.2.3.2 Biological covariates analysis

Several models were tested to assess the effect of AGP on the PK parameters. First, a linear relationship between the AGP covariate and the PK parameters was tested:

$$CL \text{ or } Vd = \theta_a + \theta_1 \cdot \frac{(X - X_{mean})}{X_{mean}} \quad (6)$$

where X is expressed as the relative deviation of the individual X from population mean X_{mean}. For AGP plasma levels (AGP_{tot}), the population mean (AGP_{mean}) was equal to 0.95 g/l. This equation (5) was also used afterwards for testing some other biological covariates (see below).

Based on a hyperbolic relationship between AGP_{tot} and CL/Vd was observed on the plots (see Results and Figure 3.7), two simple hyperbolic models (Power and E_{max}-like respectively) were also tested on CL and Vd:

$$CL \text{ or } Vd = \theta_a + AGP_{tot}^{-\theta_1} \quad (7)$$

$$CL \text{ or } Vd = \theta_a - \frac{\theta_1 \cdot AGP_{tot}}{\theta_2 + AGP_{tot}} \quad (8)$$

Additionally, a model derived from the expression of the free fraction (f_u), proposed by Rowland et al [21] ($f_u = \frac{1}{1 + K_a \cdot f_{up} \cdot AGP_{tot}}$), was assessed:

$$CL = \frac{\theta_1}{1 + \theta_2 \cdot AGP_{tot}} \quad (9)$$

where θ_1 represents the unbound clearance (CL_u) and θ_2 accounts for both the association constant (K_a) and the fraction of the number of binding sites unoccupied (f_{up}). Notice that this model assumes constant, non-saturable free fraction.

Furthermore, a mechanistic approach was built up on the basis of physiological considerations (see Figure 3.1). The model given by equation (3) was thus rewritten, postulating that only the unbound imatinib concentration C_u was able to undergo first-order elimination through an unbound clearance process:

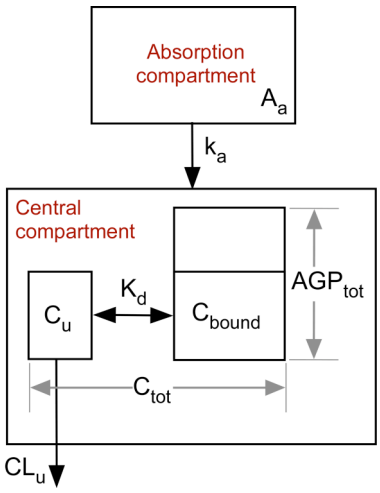

Figure 3.1. Pharmacokinetic model derived from a mechanistic approach (A_a, imatinib amount in absorption compartment; k_a, absorption constant; K_d, dissociation constant; C_u, free imatinib concentration; C_{tot}, total imatinib concentration; C_{bound}, imatinib concentration bound to AGP; AGP_{tot}, total plasma AGP levels; CL_u, free clearance)

$$\frac{dA_2}{dt} = k_a \cdot A_1 - CL_u \cdot C_u \tag{10}$$

The values of C_u were related to the predicted concentrations C_{tot} through the following equation, incorporating AGP_{tot}, the AGP dissociation constant for imatinib K_d and a scaling factor L:

$$C_u = \frac{C_{tot} - K_d - L \cdot AGP_{tot} + \sqrt{(C_{tot} - K_d - L \cdot AGP_{tot})^2 + 4 \cdot K_d \cdot C_{tot}}}{2} \tag{11}$$

The derivation of equation (11) is detailed in Appendix 3.3. A similar equation had been previously proposed for the analysis of sulfonamides PK by Bourne et al [22,23] (we simply used the dissociation constant K_d, instead of the association constant K_a). The L constant accounts for the difference in concentration unit between C_{tot} (µg/l) and AGP_{tot} (g/l), assuming a one-to-one molar binding ratio [24]. It was fixed to 11.7 (considering a molar mass of 493.6 g/mol for imatinib [25], and a mean molar mass of 42'000 g/mol for AGP [26]).

On the basis of similar considerations, another hyperbolic equation relating Vd to AGP_{tot} was derived (see Appendix 3.4 for details):

$$Vd = \frac{-\{[-K_d - L \cdot AGP_{tot} + \sqrt{C_{tot}{}^2 + 2 \cdot C_{tot} \cdot (K_d - L \cdot AGP_{tot}) + (K_d + L \cdot AGP_{tot})^2}] \cdot (V_{AGP} - V_u)\} + C_{tot} \cdot (V_{AGP} + V_u)}{2 \cdot C_{tot}} \quad (12)$$

where V_{AGP} represents the volume of distribution of the protein and V_u the volume of distribution of the unbound fraction of imatinib. Formally, equation (12) makes Vd dependent on the predicted total concentration of imatinib (C_{tot}). To fit such an equation in NONMEM®, it was however necessary to use the observed concentration instead.

The demographic covariates (BW, gender and age) and the pathology diagnosis were tested again (see equations (4) and (5)), based on the new model incorporating AGP (equations (10) and (11)).

Additionally to this AGP model, the effect of other biological covariates (MDR1 genotype, CYP3A4 activity and creatinine clearance) on imatinib PK was assessed. For MDR1, the following expression was used:

$$CL \text{ or } Vd = \theta_a + \theta_1 \cdot MDRT \quad (13)$$

where MDRT is equal to -1 for MDR1 genotype 3435CC, 0 for 3435CT and 1 for 3435TT. For CYP3A4 activity (3A4A, expressed as urinary cortisol ratio) and creatinine clearance (CLCRT), the linear equation 5 was used with $3A4A_{mean}$ = 5.4 (6β-hydroxycortisol/cortisol ratio) and $CLCRT_{mean}$ = 75 ml/min. CLCRT was calculated according to the Cockcroft and Gault formula [27].

At last, some derived parameters (elimination half-life $t_{1/2}$, absorption half-life $t_{1/2a}$ and imatinib free fraction f_u) were calculated as follows: $t_{1/2} = \frac{\ln 2 \cdot Vd}{CL}$, $t_{1/2a} = \frac{\ln 2}{k_a}$, $f_u = \frac{C_u}{C_{tot}}$, where C_u is the predicted unbound concentration and C_{tot} the predicted total concentration.

3.2.4 Statistical model

A hierarchical model was used to account for inter- and intraindividual variability. The individual PK parameters θ_j were modeled assuming a log-normal distribution among the patients and were of the general form (see also equation (1)):

$$\theta_j = \theta \cdot e^{\eta_j} \quad (14)$$

where θ is the population mean of the parameter, and η_j are independent normally distributed random effects with mean zero and variance Ω.

A proportional model was used to describe intraindividual (residual) variability of imatinib. For the generic response Y and the corresponding prediction $\hat{Y}$, the i^{th} measurement for the j^{th} individual takes the form:

$$Y_{ij} = \hat{Y} \cdot e^{\varepsilon_{ij}} \quad (15)$$

where ε_{ij} is independent normally distributed with mean zero and a variance σ^2.

3.2.5 Parameter estimation and model selection

The analysis was performed using the computer program NONMEM® (version V, with NM-TRAN version II) [11] running on a mainframe station (Sun Fire® 3800 server with UltraSPARC® III processors; Sun, Santa Clara, USA). It uses mixed (fixed and random) effects regression to estimate population means and variances of the PK parameters and to identify factors that may affect them. The data were fitted using a stepwise procedure and the first-order conditional method (FOCE INTERACTION and three significant digits) with the subroutine ADVAN 6. To determine the statistical significance between two models, different statistical selection criteria can be used that require a minimal decrease of 2 to 10 points in the objective function (OF) [28,29]. The decrease in OF corresponds to minus twice the logarithm of the linearized maximum likelihood of the model, and is approximately χ^2 distributed (based on the likelihood ratio test). Therefore, a decrease of more than 3.8 points was considered significant for one additional parameter and 5.9 points for two additional parameters. Additionally, regression diagnostic plots generated with Excel® (version 11.2, Microsoft Co., Redmond, USA) were used for comparison between models.

The general model was first evaluated with the data from the 5 patients having undergone intensive PK investigation. The analysis on the whole population was then conducted starting from those initial estimates. The influence of each recorded patient characteristics (i.e. covariates) on the Bayesian individual PK estimates of oral clearance (CL) and oral volume of distribution (Vd) was visually explored with Excel® (Appendix 3.5). The patient characteristics showing some potential influence on the PK parameters were then evaluated with NONMEM® by sequentially introducing them in the model. Finally, a simulation based on the final PK parameter estimates was performed with Excel® using first-order error propagation formulae to calculate the 90% prediction interval (Figure 3.6 and 3.8), encompassing the 5th and 95th expected concentration percentiles at each time point. The figures were generated with Prism® (version 4.03, Graphpad Software Inc., San Diego, USA).

3.3 Results

The 321 imatinib plasma concentration values measured in the 59 patients ranged between 67 and 11'221 µg/l. The population description is summarized in Table 3.1. Figure 3.2 shows the plot of the concentrations *versus* time after drug intake for all samples collected during the 3-year study. This Figure also integrates the additional measurements of the 5 patients followed at the PCL Clinical Research Unit during an entire dosing interval (9 blood samples drawn between 0 h and 8 h after drug intake). It is important to note that the analytical results of the 321 samples collected on EDTA measured were increased by 5 %, to correct for calibrations prepared in citrated plasma, in accordance with the recommendations specified in § 2.1.3.3.

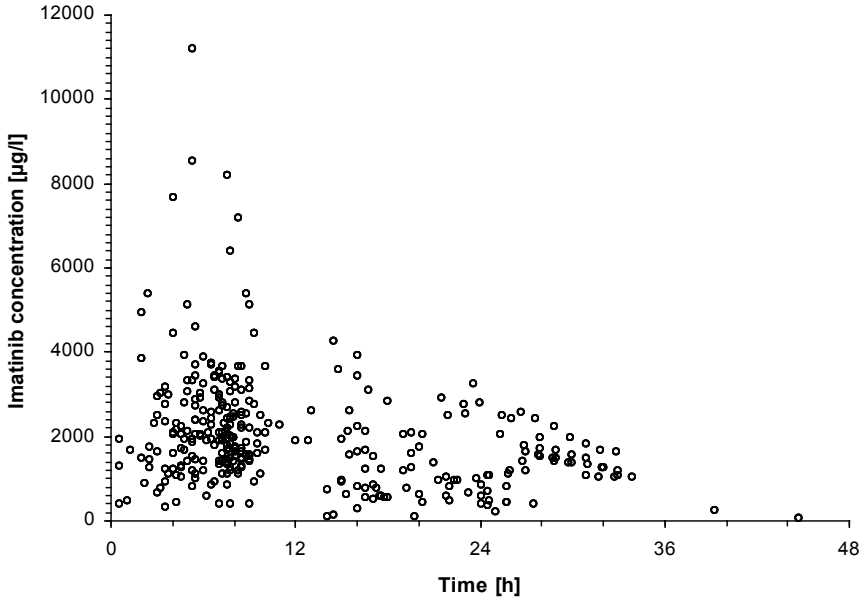

Figure 3.2. Concentration versus time plot of the 321 samples collected during the 3-year study

The P-gp polymorphism assessed in the sub-population of 36 patients (corresponding to 245 samples) revealed that 5 had the *MDR1* 3435CC and 9 had TT genotypes, theoretically associated respectively with higher and lower P-gp expression as compared to the CT genotype found in 22 patients. Figure 3.3 shows an example of one of the electrophoretic gels of the digested PCR products obtained after migration and staining with ethidium bromide.

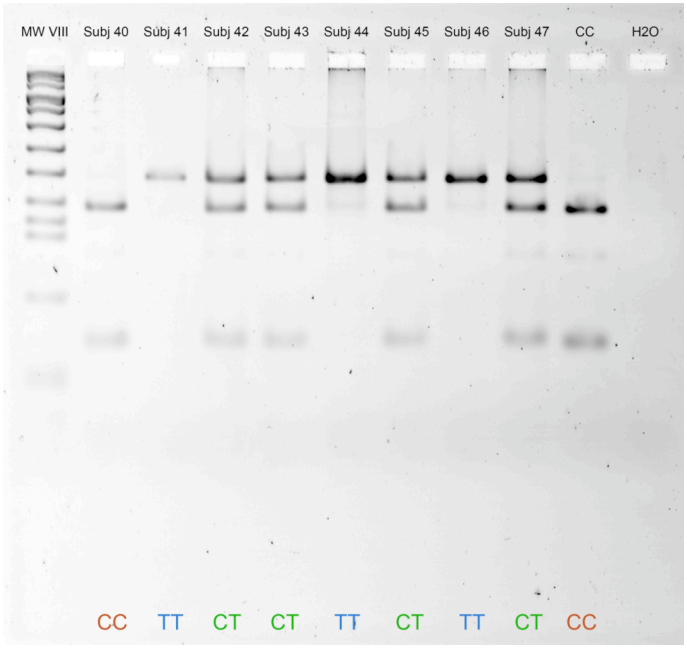

Figure 3.3. Electrophoretic gel of 8 patients (subjects 40 to 47), along with DNA molecular mass marker VIII (far left), CC control and blank control (H2O; right)

The CYP3A4 activity (evaluated as the urinary cortisol ratio and determined in 39 patients; representing 164 samples) ranged from 0.7 to 38.5 (levels assumed to reflect metabolic activity). There are still controversies as to whether the 6β-hydroxycortisol/cortisol ratio reliably reflects the CYP3A phenotype. Information available at present suggests that this ratio is probably only moderately useful to compare interindividual CYP3A4 activity in a population, whereas the ratio may certainly constitute a useful index to assess the intraindividual variability of the CYP3A phenotype of a given subject, and its evolution over time. Despite this controversy, the data were integrated into the database without *a priori*, in order not to lose any power in the analysis.

Finally, the assessment of AGP plasma concentration in 51 patients (corresponding to 278 samples) provided results ranging from 0.4 to 3.2 g/l. As for the CYP3A4 phenotype, the data were not categorized into patient subgroups (i.e. low = 0.4-1.0 g/l, intermediate = 1.0-2.0 g/l, etc).

3.3.1 PK Population model

A one-compartment model with first-order absorption from the gastrointestinal tract appropriately described the data (equations (2) and (3)), and a 2-compartment model did not improve the fit, with a difference in the objective function (ΔOF) of 0.0. In the absence of intravenous data, the mean population bioavailability (F) was fixed to 1, in accordance with the almost complete absorption reported for imatinib [30,31]. Significant inter-patient variability

could be assigned to both oral clearance (CV = 45%; ΔOF = -123.7) and oral volume of distribution (CV = 130%; ΔOF = -30.2), with a covariance between CL and Vd further reducing the objective function (ΔOF = -18.4).

3.3.1.1 Demographic covariates model

Established on this basic model, individual estimates of CL and Vd were derived for each patient and plotted *versus* all collected covariates to identify potential influences. Among the demographic covariates, body weight (BW), height, age, gender, as well as pathology diagnosis, showed some influence on imatinib PK. Height, being notably correlated to body weight, was not further investigated, assuming that the body weight was instead of interest to predict imatinib PK parameters. CL increased significantly with BW (ΔOF = -63.4). The combination of BW and gender produced a slight improvement over the model with BW alone (ΔOF = -3.5). The addition of age and pathology diagnosis provided some further improvement of the model (ΔOF = -4.5 and -4.6 respectively). Finally, gender significantly affected Vd (ΔOF = -5.1). Although gender, age and pathology diagnosis were of borderline statistical significance, we kept them in the model since they are always recorded for each patient and might be clinically relevant for selected patients (global ΔOF = -17.7).

Coadministration of CYP3A4 inhibitors (ΔOF = -0.8) or inducers (ΔOF = 0.0) did not appear to affect imatinib PK to a statistically relevant extent in this population of patients.

With the use of the Excel® program, it has been possible to generate for each step of this model development a diagnostic plot, similar to the one generated for the final demographic model retained (Figure 3.4). This latest plot shows a reasonable model that reliably fits the data. All concentration data are given in μg/l.

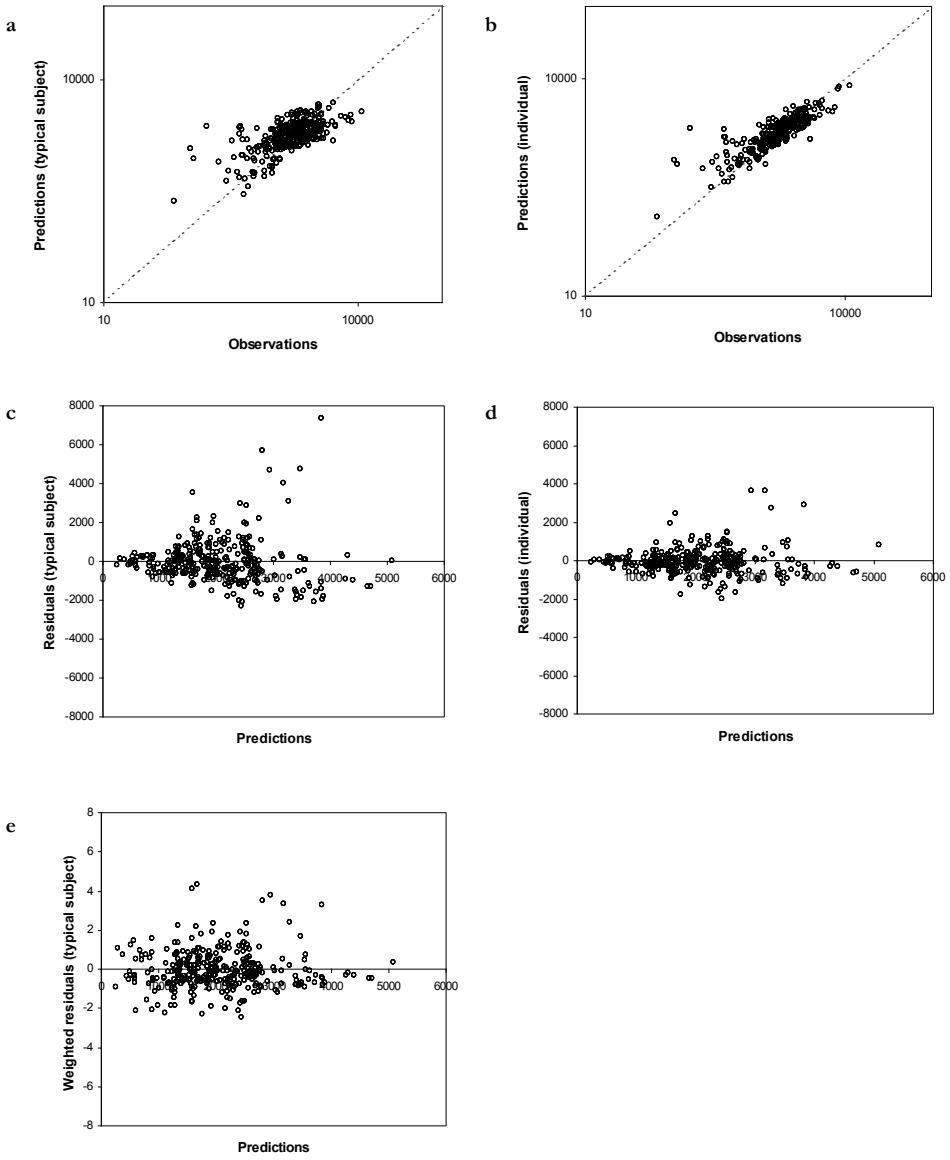

Figure 3.4. Diagnostic plots of the final demographic PK model: a) observed values *versus* population predicted values; b) observed values *versus* individual predicted values; c) population predictions *versus* population residual values (i.e. difference between observed and predicted values); d) population predictions *versus* individual residual values; e) population predictions *versus* population weighted residual values

The data of the 5 patients having participated in the whole-day study have been useful to validate the model by visual examination of the curves fitted to the individual data of these patients (see Figure 3.5 for the graphs obtained with the final model).

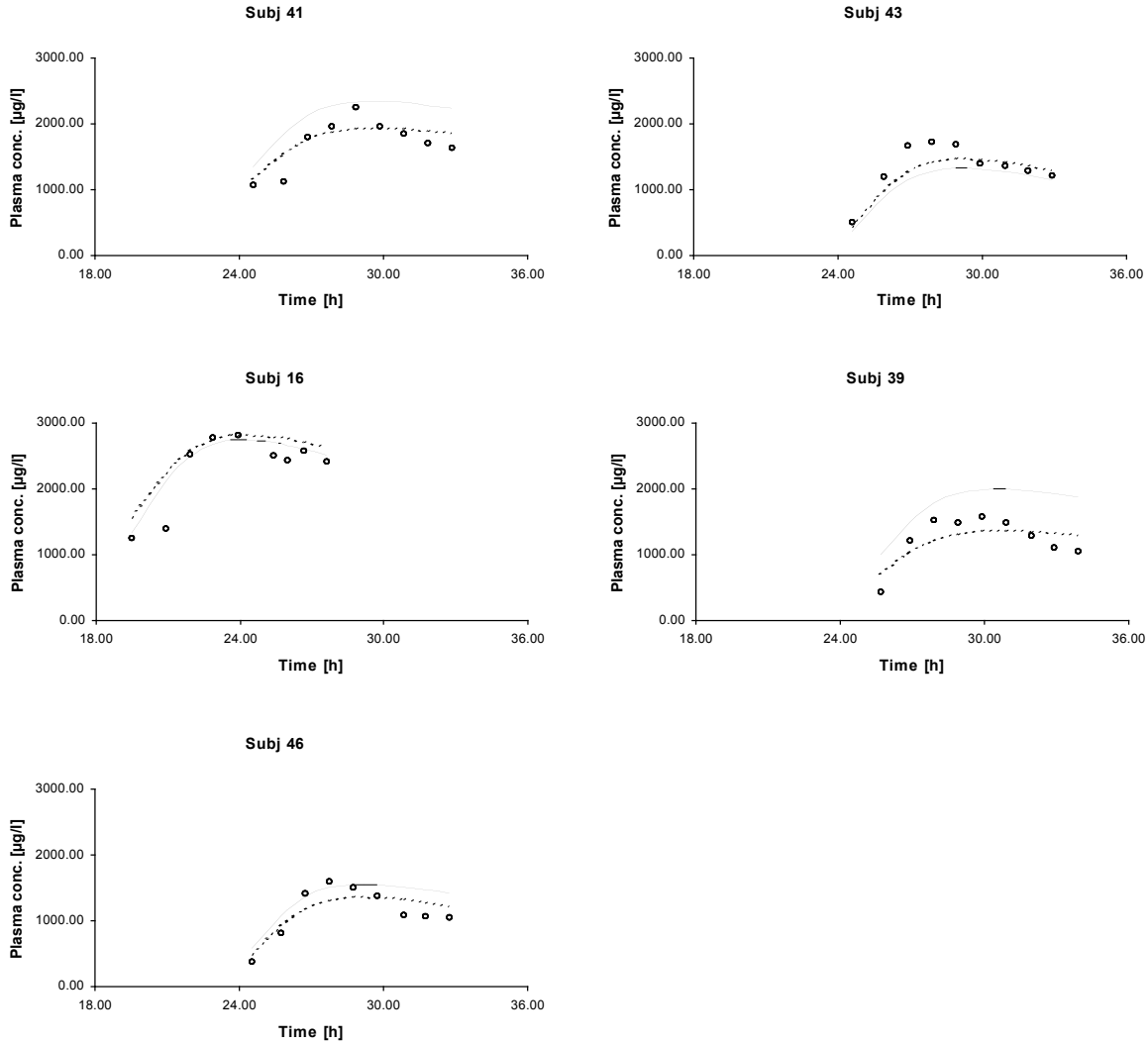

Figure 3.5. Diagnostic plots of the 5 patients participating in the whole-day PK study (continuous lines: population average prediction; dashed lines: individual maximum likelihood curve fit)

The final population estimates of the demographic model were CL 14.3 l/h, and oral Vd 347 l. The derived elimination half-life was 17 h, while the absorption half-life was 1.1 h. The interindividual variability in CL and Vd (CV = 36% and 63%) remained higher than the residual intraindividual variability (CV = 31%). CL increased by 99% on body weight doubling, decreased in female compared to male patients by 6%, decreased by 16% on age doubling, and decreased by 8% in GIST patients compared to CML patients. The building steps of this demographic model can be found in Appendix 3.6, and the PK parameter values obtained are given in Table 3.2. The observed plasma concentrations of imatinib are presented in Figure 3.6, along with the population average and 90%-prediction interval (calculated with Excel® by using the Bateman model with log-normal error propagation[3] and plotted with Graphpad Prism®).

[3] Concept developed by Dr Thierry Buclin.

Table 3.2. Population pharmacokinetic parameters of imatinib according to the two main models developed (FOCE INTERACTION method)

Model	Parameter	Population mean		Interindividual variability[1]	
		Estimate	[2]SE	Estimate	[2]SE
Demographic	CL (l/h)	14.3	7.1%	36%	28.6%[3]
	Vd (l)	347	17.9%	63%	39.6%[3]
	k_a (h^{-1})	0.61	30.0%	-	
	σ (CV %)[4]	31 %	20.3%[3]	-	
AGP	CL_u (l/h)	1310	13.1%	17%	40.7%[3]
	Vd (l)	301	7.8%	66%	46.5%[3]
	k_a (h^{-1})	0.61	18.9%	-	
	K_d (mg/l)	0.090	14.3%	-	
	σ (CV %)[4]	23%	20.3%[3]	-	

[1] Estimates of variability expressed as coefficient of variation (CV%)
[2] SE, standard error of the estimates, expressed as CV%
[3] SE, standard error of the variance components, taken as $SE_{estimate}$ / estimate, expressed as a percentage
[4] Residual intraindividual variability of the plasma concentration, expressed as CV%

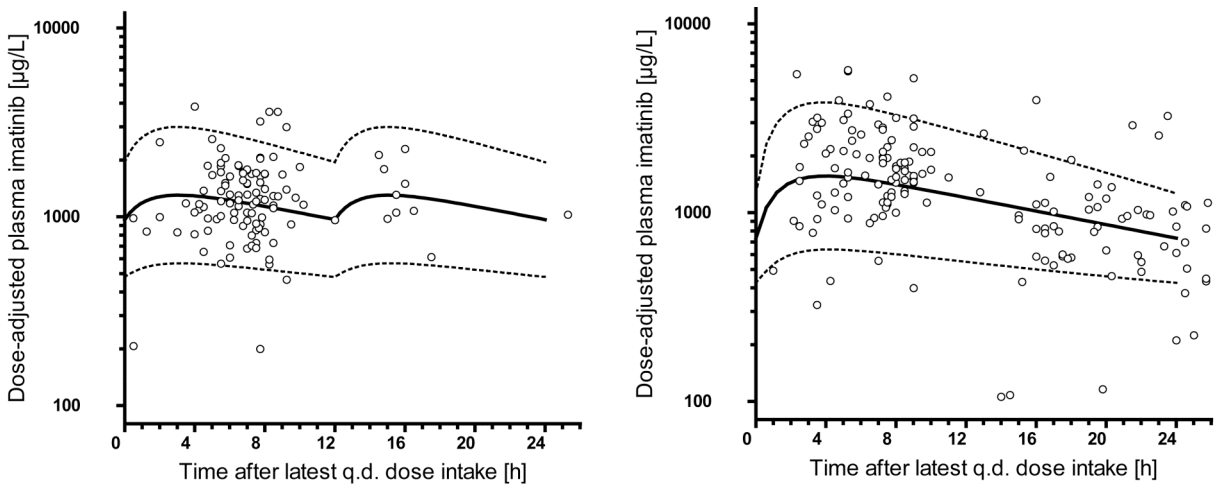

Figure 3.6. Imatinib plasma concentration observed in patients receiving imatinib, along with the average population prediction (solid line) and the 90% prediction interval (dashed lines). The graphs represent a once (right-hand part) and a twice (left-hand part) daily regimen, based on the demographic covariates model

3.3.1.2 Biological covariates analysis

The plots from Figure 3.7 (left panel) clearly suggest a hyperbolic dependency of imatinib oral CL and Vd on plasma AGP levels. The use of a linear model to characterize the relationship between CL and AGP (equation (6)) improved significantly the fit (ΔOF = -170.5). Only a slightly better description of the data was detected when using a power function (equation (7); ΔOF = -5.6). The use of an E_{max} model (equation (8)) allowed an important improvement of the fit (ΔOF = -267.2), and the constant free fraction approach of equation (9) an even better fit (ΔOF = -271.4). Finally, the mechanistic approach (equations (10) and (11)) provided the best improvement of the fit (ΔOF = -284.1), and the plot of CL_u *versus* AGP_{tot} showed an absence of a

relationship between these two variables (see Figure 3.7, right panel). As the apparent volume of distribution calculated with this model still showed some correlation with AGP_{tot}, we tried to model the influence of AGP plasma levels on Vd (using equations (6), (7), (8) and (12)). The most significant improvement was obtained using a linear relationship (equation (6); ΔOF = -11.7). The E_{max}-like relationship brought no significant improvement of the fit (equation (8); ΔOF = -2.2). Finally, the mechanistic approach (equation (12)) was not superior to the model not taking into account AGP (ΔOF = -0.0). This was probably due to the lack of available data with very low AGP plasma levels that could have accounted for a residual hyperbolic relationship between AGP_{tot} and Vd. As this mechanistic model could not be retained, Figure 3.7 (right panel) represents the relationship between AGP_{tot} and total volume of distribution (Vd).

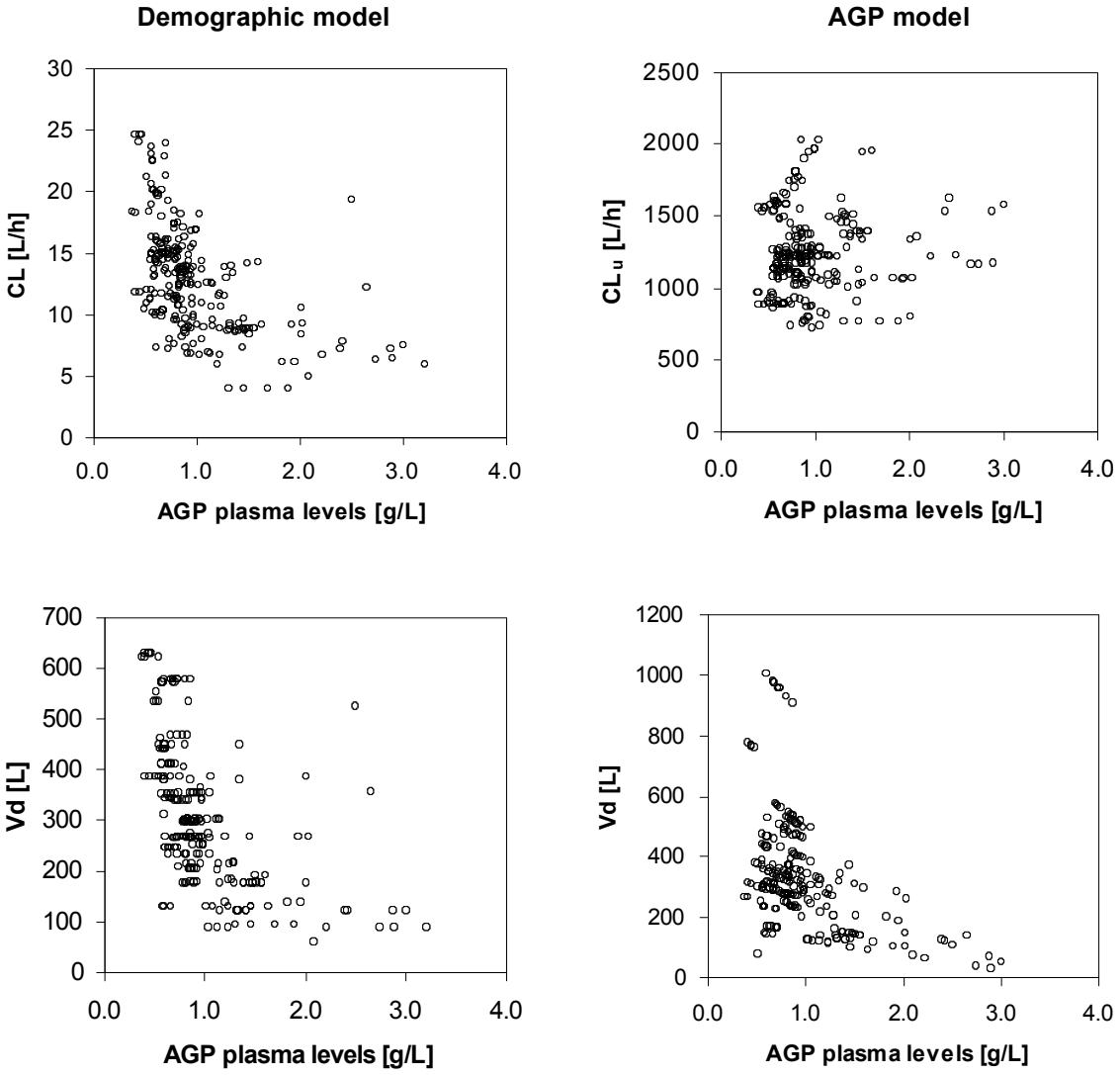

Figure 3.7. Pharmacokinetic parameters estimates derived from the demographic covariates model (left panel) and from the final AGP model (right panel), plotted according to AGP plasma levels

Following the inclusion of AGP in the analysis (equations (10) and (11)), the four demographic covariates, found significantly correlated with CL in the previous analysis (see above), were again added into the AGP model, to affect CL_u (rather than on CL) and Vd (linearly correlated to AGP_{tot} by equation (5)). While BW still improved the prediction of CL_u (ΔOF = -27.0; change by 91% on body weight doubling), the addition of age and gender did not significantly improve the model as compared to BW alone (ΔOF = -4.7). At last, pathology diagnosis appeared to have no significant effect on Vd (ΔOF = -0.5), but significantly decreased CL_u in GIST patients (ΔOF = -6.8; difference of 10.2% between GIST and CML patients), confirming the effect already observed with the demographic covariates model.

Finally, the influence of three other biological covariates were assessed. *MDR1* polymorphism was tested in the sub-population of 36 patients for whom both *MDR1* genotype and AGP plasma levels were available (corresponding to 245 samples). The assignment of this genotype as a covariate on either CL_u or Vd tended to improve the fit (ΔOF = -1.3 and -1.0), albeit not significantly. As compared to the *MDR1* 3435CC genotype, CL_u decreased by 10.6% for the TT genotype, while Vd decreased by 29.8%. No clear influence of CYP3A4 activity on CL_u or Vd could be detected (ΔOF = -0.2 and -1.0) in this limited number of observations (154 samples in 39 patients). CYP3A4 activity tended to increase CL_u by only 1.1% when the marker value doubled. Creatinine clearance significantly influenced CL_u (ΔOF = -5.7; change of CL_u by 29.0% on creatinine clearance doubling) and Vd (ΔOF = -7.0; change of Vd by 53.2% on creatinine clearance doubling), in the corresponding subpopulation (271 samples in 47 patients). Such findings were in line with those obtained with the demographic model (data not shown).

The final parameters of the AGP model are given in Table 3.2, and Figure 3.8 presents the modeled free plasma concentrations of imatinib, along with the population average and 90%-prediction interval. The *in vivo* dissociation constant (K_d) of $8.97 \cdot 10^{-2}$ mg/l (i.e. $1.82 \cdot 10^{-4}$ mmol/l), corresponds to an association constant of $5.5 \cdot 10^{6}$ l/mol and leads to a median free fraction (f_u) of 1.1% (range 0.3-2.3). The AGP model building steps can be found in Appendix 3.7.

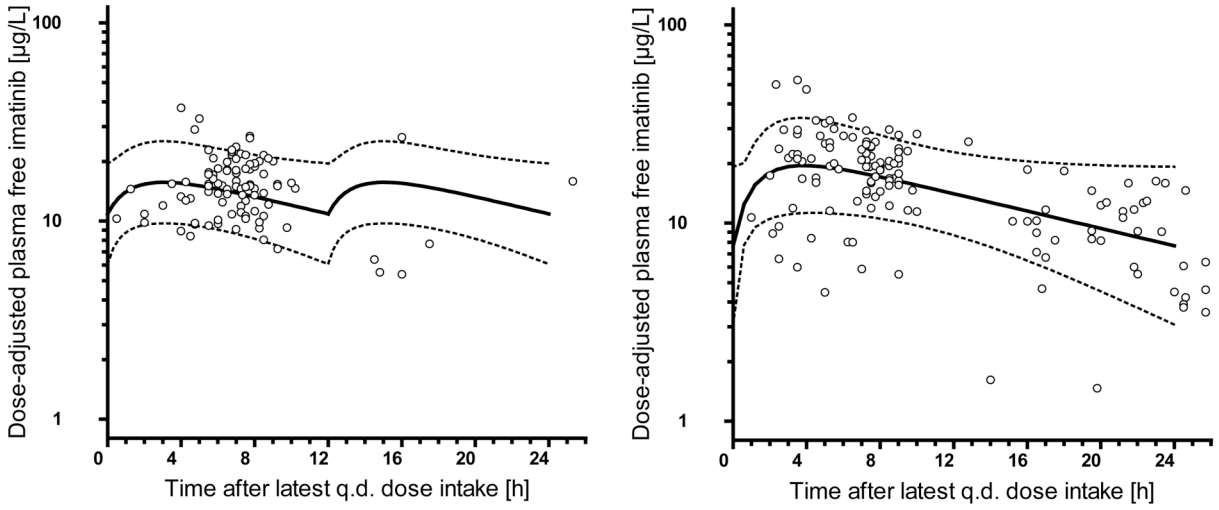

Figure 3.8. Free imatinib concentration calculated in patients receiving imatinib, along with the average population prediction (solid line) and the 90% prediction interval (dashed lines). The graphs represent a once (right-hand part) and a twice (left-hand part) daily regimen, based on the AGP covariates model

3.3.2 Intracellular Pharmacokinetics

Figure 3.9 shows the intracellular imatinib concentration *versus* time profiles, with the corresponding plasma profiles, observed during the whole-day study carried out for the 5 consenting patients studied intensively.

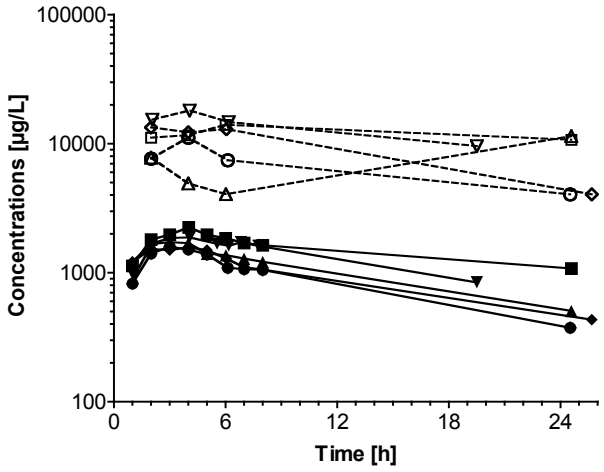

Figure 3.9. Intracellular and plasma pharmacokinetic profile of imatinib in the 5 patients of the whole-day study (each shape represents a different patient; with solid lines: plasma levels; broken lines: PBMC levels)

As these patients were at steady state on a once-daily regimen, the levels at 0 h (i.e. prior to dose intake) were plotted at a time corresponding to the duration elapsed between the previous drug intake (i.e. the day before) and the 0 h sampling. This extrapolation allows displaying the entire pharmacokinetic profile of imatinib over 24 h. However, this approximation was only done for the graphical presentation and was not taken into account for the NONMEM analysis (into

which the plasma concentrations were incorporated). Except for one subject, the intra/extracellular ratio appeared rather constant, and indicated an average 8-fold accumulation of imatinib within cells as compared to plasma levels (Figure 3.10). The atypical appearance of the intracellular curve in the subject represented by open triangle shapes is most probably due to inaccurate cell counting in the sample corresponding to the 24 h-level.

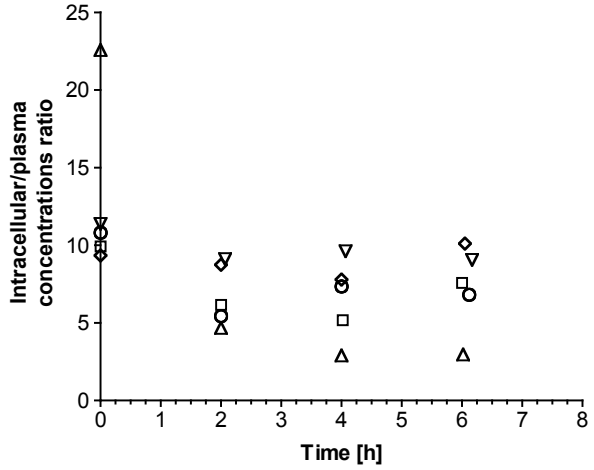

Figure 3.10. Evolution of intracellular/plasma concentrations ratio over time in the 5 patients of the whole-day study (each shape represents a different patient)

3.4 Discussion

This study enabled the development of two main types of imatinib population PK models. The use of a demographic covariates model confirms the large interindividual variability characterizing imatinib disposition, which can only be partly explained by the considered covariates (body weight, gender, age and pathology diagnosis). Together with the residual intra-patient variability (31%), this translates into an average 5-fold width of the prediction interval around the mean PK curve (or 6-fold at C_{max}; Figure 3.6). Our estimates of CL and Vd are in good agreement with the study values recently published in GIST [4] and CML [2] patients, as well as in healthy volunteers after a single oral dose [6]. These results also confirm that pathology diagnosis has only a small clinical impact on total and free clearance, in agreement with the manufacturer initial assumption of unchanged kinetics according to the treated disease [7]. The elimination half-life of 17 h is also in good accordance with previously reported values of 17-19 h in CML patients [1,3,30], 12-16 h in GIST patients [4], and 14 h in healthy volunteers [6]. The relationship between CL and BW suggests that it could be useful to adapt imatinib dosage to patient body weight. Moreover, the important variability observed implies that a given dose of imatinib may yield, in selected patients, circulating concentrations departing markedly from those

expected based on the average PK profile (established in stringent phase I and II clinical studies). This variability represents one argument in favor of individualizing imatinib dose prescription based on a therapeutic drug monitoring (TDM) program. Figure 3.6 provides a first typical concentration profile usable for Bayesian-type dosage adjustment in patients receiving imatinib.

Unlike other previously studied covariates shown to have only a modest influence, AGP plasma levels proved to have a marked impact on imatinib PK. AGP plasma levels explained indeed about one half of the inter-patient variability in total CL. With the model incorporating this biological covariates, the estimates of Vd and k_a remained similar, but the 90%-prediction interval around the mean PK curve was reduced to a 3-fold width (Figure 3.8). We thus report here the first model of the formal impact of AGP plasma levels on imatinib total plasma PK. This model is derived from mechanistic considerations assuming a saturable protein binding, because of the limited amount of circulating carrier. The disappearance of the effects of gender and age in this AGP model can probably be explained by the known intercorrelation of age and gender with circulating AGP levels [32]. A role of AGP on imatinib disposition has already been discussed [5,33], and the marked relationship observed between protein binding and imatinib PK profile might raise again the question as to whether high levels of AGP might represent a resistance factor [34-36]. However, imatinib being a drug of low hepatic extraction, a change in protein binding should not translate into significant variations in free drug concentration, since CL_u remains constant as assumed in our model. This implies that the free AUC, and thus the cellular exposition to the drug, remains unaffected. Furthermore, it is worth noting that one might expect AGP levels to fall in line with the response to imatinib treatment, as the disease burden is reduced.

On the other hand, as protein binding affects the total concentration of the drug, it would represent an important issue to take into account when monitoring and interpreting total concentrations. Our findings indeed indicate that total plasma imatinib levels do not simply reflect the free (and thus the target) concentrations of this molecule [33]. Would imatinib be accepted as a drug requiring TDM, either the measurement of free concentration or the correction of the total concentration by the actual AGP plasma level should be considered for a precise interpretation of the results. Although free drug concentration monitoring might be more appropriate, the technology to do so is not widely available (we are however considering the development of a liquid chromatography-tandem mass spectrometry method). Using AGP plasma levels instead and deducing the free fraction of the drug could thus represent a more convenient approach. In day-to-day practice, the determination of total AGP plasma level and

total imatinib concentration in a patient would enable to calculate a free concentration of imatinib using the equation (11) above. This value could then easily be compared to the typical free concentration profile given in Figure 3.8 and provide useful information for clinicians regarding a suitable dose adjustment to reach an adequate range of concentration. By combining Figures 3.6 and 3.8, we have obtained Figure 3.11, which gives a good illustration of the difference between the two approaches (total *vs* free concentrations). This summarizing representation was presented in two recent meetings [37,38].

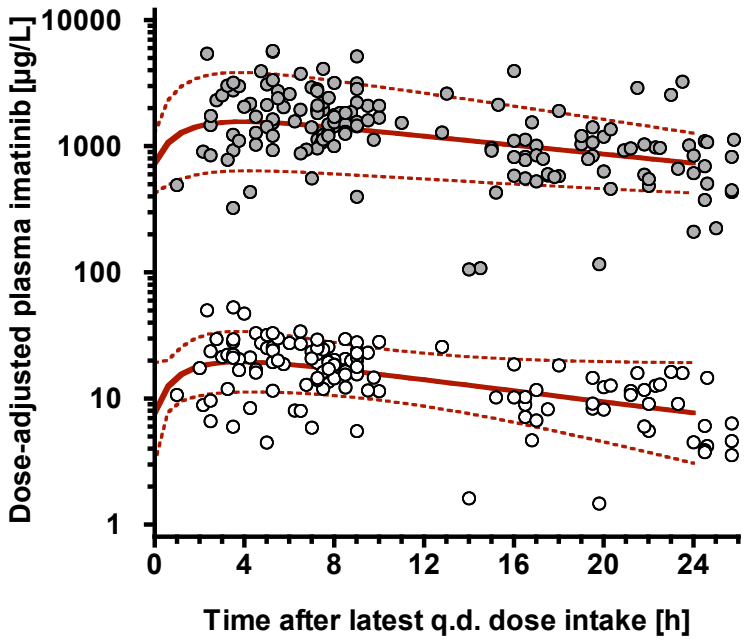

Figure 3.11. Comparison of imatinib plasma concentrations in patients receiving imatinib, along with the average population prediction (solid line) and the 90% prediction interval (dashed lines). The upper part represents the observed C_{tot} and the lower part the estimated C_u

Our model incorporating AGP also provides interesting estimates of the *in vivo* dissociation (respectively association) constant and f_u, regarding this protein binding. Our association constant of $5.5 \cdot 10^6$ l/mol determined *in vivo* is not very different from the value of $4.9 \cdot 10^6$ l/mol previously reported *in vitro* [24]. This denotes a very high affinity of imatinib for AGP, and this could explain the marked effect observed for this carrier, as previously reported for other anticancer agents [39]. The median *in vivo* free fraction (f_u) of 1.1% is of the same order of magnitude as the value determined *in vitro* (3.1% at AGP concentration of 1 g/l and imatinib concentration of 0.3-0.5 µg/ml [40]). Such findings clarify in part the *in vivo* impact of protein binding on imatinib disposition, as wished by Peng *et al* in a recently published review [5]. The effect of albumin could also have been assessed. However, as the concentration of this protein varies much less than the one of AGP, and as imatinib is known to bind preferentially to

AGP [24,40]), albumin level has not been considered in our model. A recent population pharmacokinetic study did not observe any significant relationship between albumin levels and imatinib PK parameters [4].

Concerning other biological covariates, *MDR1* polymorphism and CYP3A4 function seem to have a limited impact on imatinib disposition, even the 3435C>T SNP known to be correlated to imatinib AUC [8]. The use of other CYP3A4 markers, such as erythromycin breath test or midazolam clearance, may help to define better any relationship between imatinib pharmacokinetics and the activity of this enzyme, as recently demonstrated [8]. However, the use of such exogenous markers was beyond the scope of our exploratory study. Despite the controversial validity of the urinary cortisol ratio test used, it has the advantage to represent a non-invasive test [41]. The lack of apparent effect of CYP3A4 inhibitors or inducers is probably due to the limited number of patients exposed to such drugs in our population. Most CML and GIST patients were actually outpatients without comedications. On the other hand, the observed effect of creatinine clearance on imatinib clearance and volume of distribution might suggest some influence, either direct or indirect, of renal function on imatinib disposition, despite its predominant hepatic clearance [30].

In addition, this study provides the first *in vivo* assessment of imatinib intracellular accumulation. The average 8-fold cell/plasma ratio determined in samples taken simultaneously is in agreement with the available *in vitro* data from the literature (approximate 5-fold factor in leukemia cell cultures incubated with imatinib and human plasma [3]). This is in line with the recent demonstration that imatinib undergoes active transport *inside* leukemia cells by the solute liquid transporter hOCT1 [42], which may have functional and clinical consequences. Interestingly, it has been recently reported that hOCT1 expression level varies between responders and non-responders CML patients [43]. The clinical relevance of those observations certainly deserves further evaluation. The relative stability of the cell/plasma ratio suggests though that measuring plasma concentration (either total, AGP-corrected or free, as discussed above) probably provides a good surrogate for intracellular exposure. It is noteworthy that intracellular concentration of imatinib should likely be better termed "cell-associated amount", as the drug in its cellular environment may be embedded in membrane lipid bilayers, complexed to cytoplasmic proteins or sequestrated in specific subcellular fractions. This has been reported for instance with anti-HIV drugs [44,45], and only a fraction of the so-called "intracellular amount" of the drug is actually available to exert its pharmacological activity. It is also worth noting that during the course of the study, pathologists have sent us, at several occasions, some GIST tumors

biopsy specimens taken from patients on imatinib. Thus, the setting-up of an analytical method allowing the intra-tissular measurement of imatinib is anticipated before its application to the analysis of these samples, as well as of those further collected. This would allow comparing the intra-tumoral imatinib penetration in responding or non-responding GIST patients.

In conclusion, the high inter-patient and rather limited intra-patient variability in imatinib PK (along with the potential relationship of exposure with treatment efficacy and toxicity suggested by some studies and case reports mentioned above) are arguments to further investigate the potential usefulness of a TDM program for this drug. Taking into account protein binding may represent an advantage over the rough measurement of total plasma concentration, but protein binding may not represent by itself a resistance mechanism. The monitoring of imatinib exposure, with consequent dosage individualization, might play an appreciable role in limiting the incidence of side effects and delaying the emergence of tumor resistance, which seems to be favored by prolonged subtherapeutic drug levels exposure both *in vitro* [46,47] and *in vivo* [48]. Similar considerations may apply to other signal transduction inhibitors currently under development for the treatment of several malignancies. In addition, devoting attention to TDM could contribute to optimize the patients' compliance, considered as an important issue for sustained inhibition of target proteins. However, before individualization of imatinib therapy based on routine drug concentration monitoring can be recommended, further PK-PD analysis in well-controlled trials is required.

In that perspective, the next chapter will give some insights of a preliminary analysis of the PK-PD relationships of imatinib in our patient population.

3.5 References

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Chapter 4 Concentration-effect relationships

Complementary to the population pharmacokinetic analysis presented in Chapter 3, an exploration of pharmacokinetic-pharmacodynamic (PK-PD) relationships was carried out and is presented in the following sections.

As early as 2004, a Phase I trial in CML patients [1] had already shown that an imatinib dose of 350 mg or higher was necessary to achieve a median through level of 570 ng/ml (i.e. about 1 μM) in the studied patients, but was associated, however, with a very high variability (SD = ± 369 ng/ml). It exceeds the 50% inhibitory concentration (IC_{50}) required to inhibit *in vitro* proliferation of Bcr-Abl leukemic cells (i.e. 0.025 μM and 0.25 μM for inhibition of Bcr-Abl substrate phosphorylation and specific tyrosine phosphorylation, respectively [2]). This through plasma concentration, and the time during which imatinib plasma levels were above this level, appeared to be related to the initial PD effects of imatinib [1]. The sensitivity of Ph+ cell lines derived from patients with CML or ALL was subsequently tested. In most of these lines, the IC_{50} values were also comprised between 0.1 and 0.5 μM [3]. A target concentration of 1 μM seems thus appropriate to maximize the efficacy in CML cells and to limit the toxicity in normal mononuclear cells. However, there is still a controversy on whether this concentration does not really also affect normal mononuclear cells [4]. Alternately, such a concentration appears to be ineffective to obtain fully *BCR-ABL*-free colonies *in vitro* [4]. Moreover, no considerations about adjustments of concentrations regarding protein binding (*in vitro*, as well as *in vivo*) have been mentioned in these studies.

More information on PK-PD relationships between total –and free– plasma levels of imatinib and clinical response and toxicity seems thus needed. The present chapter will therefore present a first data analysis relating imatinib pharmacokinetics to clinical effects in our patient population. Demonstration of a clear PK-PD relationship for a drug is indeed a prerequisite to apply a therapeutic drug monitoring (TDM) approach in the management of a treatment.

4.1 Theoretical introduction

4.1.1 PK-PD relationships

Pharmacodynamics has been defined as the study of the biologic effects resulting from the interaction between drugs and biological systems [5]. Pharmacodynamics can be considered as

"what the drug does to the body", whereas pharmacokinetics is "what the body does to the drug" [6]. Figure 4.1 is an illustration of how pharmacokinetics and pharmacodynamics determine the observed pharmacological effect of a drug [5,7].

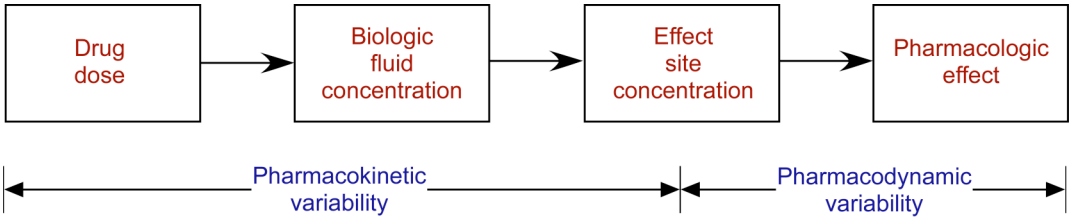

Figure 4.1. Pharmacokinetics and pharmacodynamics as determinant of the dose-response relationship

This Figure also shows that investigating only the pharmacokinetics of a drug, without assessing the pharmacological effects (efficacy and toxicity; associated with the biologic concentration) has limited clinical relevance. Since the late 1970s, there has thus been an increased emphasis on combining pharmacokinetics (PK) and pharmacodynamics (PD) in the clinical evaluation of new drugs. Appropriate analysis linking PK and PD information provides a rational basis for evaluating the impact of different dosage regimens on the course of pharmacological response [6], and is also essential in the evaluation of a TDM approach.

4.1.2 Logistic regression analysis

Since the late 1960s, the logistic regression model has become the standard method for regression analysis of dichotomous data in many fields, especially in the health sciences. Regression methods have become an integral component of any data analysis describing the relationship between a response variable (usually discrete) and one or more explanatory variables. What distinguishes a logistic regression model from the linear regression model is that the outcome variable in logistic regression is binary or dichotomous (i.e. $y = 0$ or 1) [8]. Logistic regression can also be generalized to situations where the response variable has more than two ordered response categories (i.e. $y_1, \ldots, y_i$) by considering these categories as resulting from thresholding an unobserved continuous variable u at a number of cut-points [9].

In PK-PD relationships, the response variable (efficacy or toxicity) typically belongs to the latter example. This is why logistic regression is often chosen to analyze the relationship between PK and PD variables.

4.2 Materials and methods

As previously mentioned in § 3.2.1, a comprehensive set of clinical data has been collected for all patients who participated in the clinical pharmacokinetic study. In addition to accurate dosing and sampling time information, the following data were also recorded: body weight, gender, age, height, creatininemia (CRT, in μmol/l), presence of liver or cardiac impairment, and all concomitant medication intake (Table 3.1). Moreover, the current clinical response was determined using a 3-point scale for CML/ALL (complete=2, partial=1 and absent=0 hematological remission), and according to RECIST criteria for GIST [10]. For the efficacy analysis, the RECIST criteria were afterwards recoded to match the CML evaluation (i.e. complete response=2, partial response or stable disease=1 and progressive disease=0). A standardized evaluation of the typical side effects was performed, and the number of side effects experienced by patient at each sampling time was summarized in a 4-point scale (0, 1, 2 and 3 or more side effects).

4.2.1 Standard analysis

Once the NONMEM modeling completed, individual *post hoc* Bayesian estimates of the PK parameters could be derived for all patients, and were used to calculate the individual drug exposure, expressed as AUC (area under curve; defined as $\text{Dose}/\text{CL}\cdot\tau$, where τ is the dosing interval). Associations between Dose, AUC or CL and therapeutic response (coded on the 0 to 2 scale described above), as well as the association with tolerability (count of 0, 1, 2 or 3 and more adverse effects), were explored by ordered logistic regression. Beside this per-sample analysis, a per-patient analysis was carried out with all different data collapsed in one value for each patient (i.e. average Dose, AUC and CL, and median efficacy and side effect scores). This analysis was carried out using both total plasma and free AUC and CL, given by the demographic and AGP models, respectively. The results were considered statistically significant at $p < 0.05$, while $p \leq 0.1$ values were regarded as indicative of possible trends. All statistical tests were performed with the Stata® software (version 9.1, Stata Co., College Station, USA).

4.2.2 Analysis incorporating the target mutation profile

The same above-mentioned approach was used for assessing the influence of the target mutation profile in the pharmacodynamics, by incorporating additionally the patient genotypic profile, coded on a binary scale (i.e. 1 = presence of mutation known to confer resistance to imatinib treatment, and 0 = absence of such mutation).

4.2.2.1 CML patients

The mutation profiles of *BCR-ABL* in our CML patients have been determined by InPheno (Basel, Switzerland), a collaborating spin-off laboratory of the University of Basel, which analyzed our frozen cell pellets samples.

The RNA from the samples was extracted with a phenol-containing reagent (TRI Reagent®, Sigma-Aldrich, Buchs, Switzerland). The biological sample was homogenized in the reagent and the simultaneous isolation of RNA, DNA and proteins was carried out in a single step by a liquid-phase separation. However, it was not anticipated at the initiation of our study to analyze the RNA from our patients, and the blood cellular pellet samples collected during the clinical study were frozen without cryoprotective agent (e.g. DMSO). This prevented thus InPheno to amplify the RNA corresponding to the *BCR-ABL* gene required for the subsequent cloning of the cDNA of this gene. It was thus decided to rather amplify only two exons of this gene directly from the DNA fraction. The exons chosen were exon 4 and exon 6 of the *ABL* moiety [11], containing most of the known mutations and the most frequent mutations (namely E225K/V for exon 4, and T315I and M351T for exon 6; Figure 4.2). Various reagents were mixed in the following order to prepare the PCR mix: 5.0 µl incubation buffer x 10, 1.0 µl nucleotides 10 mM (dNTPs), 2.0 µl primers (F and R for exon 4 and 6, respectively), 0.5 µl Hot Star Taq DNA polymerase from Qiagen (Basel, Switzerland), and water to 50 µl (all quantities for one sample). The mix was vortexed and aliquots were distributed in PCR tubes. The primers sequences were as follows: forward (F4) 5'-CTCTGTCCTGTGTGGAGAGCT-3' and reverse (R4) 5'-CATCG CCTAATGCCAGCAGACG-3'; and forward (F6) 5'-GAGCACAGTCTCAGGATGCAGG-3' and reverse (R6) 5'-CCAAGTGTTGCCAGCACTGAG-3'. PCR amplification was carried out with the program described in Table 4.1, using a PCR Sprint cycler (Catalys, Wallisellen, Switzerland).

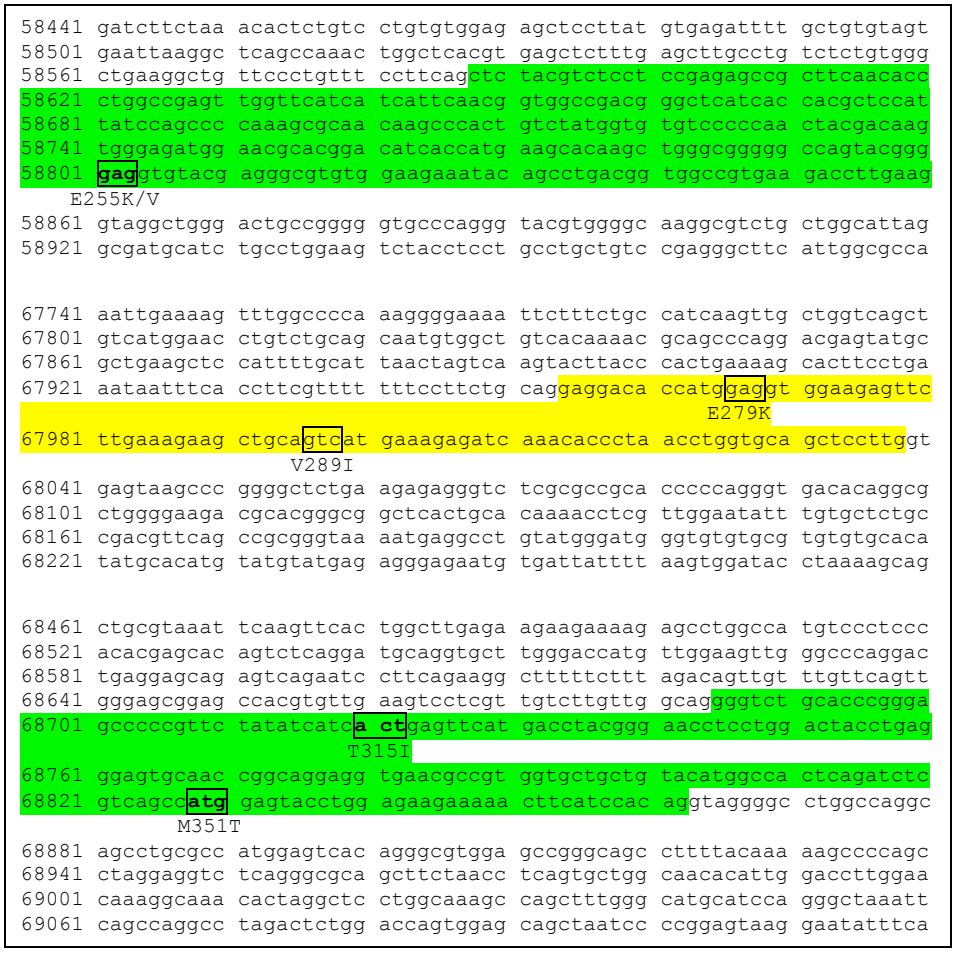

Figure 4.2. Regions chosen to design primers to amplify Abl genomic DNA (exon 4 = green, exon 5 = yellow, exon 6 = green; courtesy of Roten T, InPheno, Basel)

Table 4.1. PCR Sprint cycler program

Phases	Cycles #	T [°C]	Duration	Purpose
1	1	95	15 min	Activation step
2	30	95	30 s	Denaturation
		55	30 s	Hybridation
		72	1 min 10 s	Elongation
3	1	72	10 min	Final extension

Following this amplification, the DNA amplicons were sequenced with a 3730 DNA Analyzer (Applied Biosystems, Foster City, USA), a fully automated system for medium-to-high throughput genetic analysis. This step was performed using the ABI Big-Dye® Terminator chemistry (Applied Biosystems, Foster City, USA). The patient sequences were finally compared to the respective typical exon sequence, using the dot plots method [12] of CLC Combined Workbench (version 1.0, CLC bio A/S, Aarhus, Denmark).

It was planned that the patients presenting any mutation on one of the two exons would be attributed the mutation code 0 and those with wild-type *ABL* would be attributed the mutation code 1 (see above and § 1.4.1).

4.2.2.2 GIST patients

As some of the GIST patients (20/38) followed in Lausanne were selected in a tertiary reference center as part of the multicentric EORTC Soft Tissue and Bone Sarcoma trial, their tumor genetic profiles were available. Genomic DNA was extracted from sections of paraffin-embedded tumor blocks. Exons 9, 11, 13 and 17 of the *KIT* gene were amplified by PCR, and the amplicons were analyzed for mutations by a combination of DHPLC prescreening (WAVE DHPLC system, Transgenomic, UK) and bidirectional sequencing [13]. Specimens that had no detectable *KIT* mutation (*KIT* wild-type) were further tested for *PDGFRA* exons 12 and 18 mutations.

The patients presenting mutation on exon 11 were attributed the mutation code 1 and those presenting mutation on exon 9 or those that were wild-type were attributed the mutation code 0 (see above and § 1.4.1).

4.3 Results

4.3.1 Standard analysis

Using Bayesian PK estimates from the demographic covariates model, a pharmacodynamic exploration revealed an inverse relationship between Dose (as well as AUC) and therapeutic response ($p < 0.005$ in per-sample analysis, non-significant in per-patient analysis), with disease progression patients receiving higher doses than good responders. Similarly, a better response was observed with increasing CL ($p < 0.005$ in per-sample analysis, non-significant in per-patient analysis). It is noteworthy that Dose and AUC were highly correlated ($p < 0.005$) and the type of pathology alone was in fact sufficient to predict the response (better scores in CML patient, $p < 0.005$). A similar analysis carried out on side effects' scores showed that Dose and AUC were positively correlated with the amount of side effects ($p < 0.005$ in per-sample analysis, non-significant in per-patient analysis), but this was not the case for CL.

Using the exposure estimates derived from the AGP model (i.e. *free* drug exposure) made the relationship between free AUC and response become positive, albeit non-significant (in per-sample and per-patient analyses). Concerning the tolerability to the drug, free AUC remained positively correlated with the amount of side effects ($p < 0.005$ in per-sample, and $p < 0.05$ per-patient analyses; Stata® generated Figure 4.3, left-hand part). In the same analysis, free clearance (CL_u) showed some association with tolerability scores ($p < 0.05$ in per-sample and per-patient analysis; Stata® generated Figure 4.3, right-hand part). Interestingly, a multivariate regression

including Dose and CL_u still showed significant effects for both parameters ($p < 0.005$ in per-sample analysis and $p < 0.05$ in per-patient analysis).

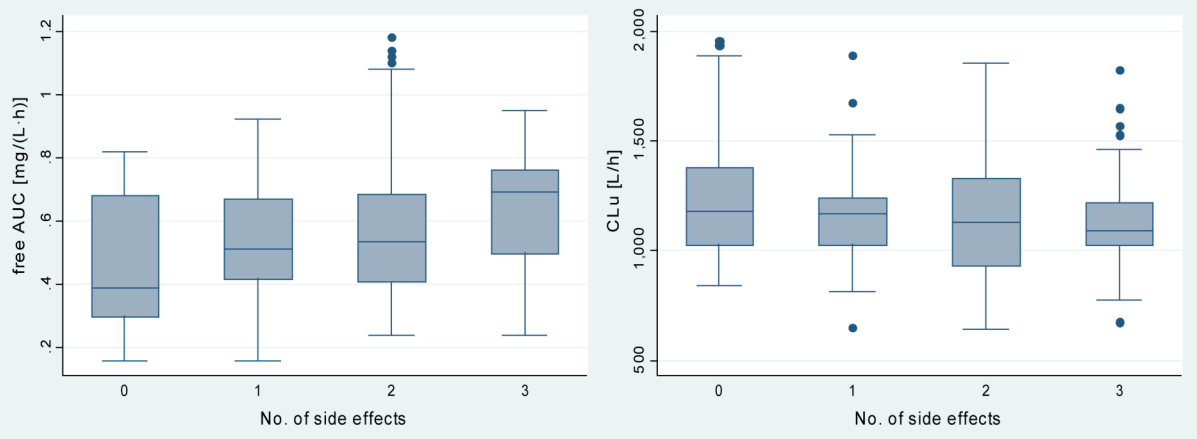

Figure 4.3. Box plot of the pharmacokinetic parameters according to side effects (0 = no side effects, 1 = 1 side effect, 2 = 2 side effects, and 3 = 3 or more side effects)

4.3.2 Analysis including the target mutation profile

First of all, Table 4.2 presents the results of all the genotypic analysis performed in our patient population and Figure 4.4 shows an example of a DNA sequence determined at InPheno.

Table 4.2. Genotypic profile of our patient population (1 = mutation profile known to confer lower response to imatinib; 0 = no deleterious mutations; - = no mutation assessment)

Patients	**Profile**	***n* (%)**
CML/ALL patients		
No. 38, 41, 105, 106	-	4 (7)
No. 33-35, 37, 39-40, 42, 44, 46-47, 51-52, 101-104, 107	0	17 (29)
None	1	0 (0)
GIST patients		
No. 6, 8-10, 12, 14, 16, 18-19, 24, 29, 31, 36, 43, 45, 48-50	-	18 (31)
No. 2-5, 7, 11, 15, 17, 21, 25-28	0	13 (22)
No. 1, 13, 20, 22-23, 30, 32	1	7 (12)

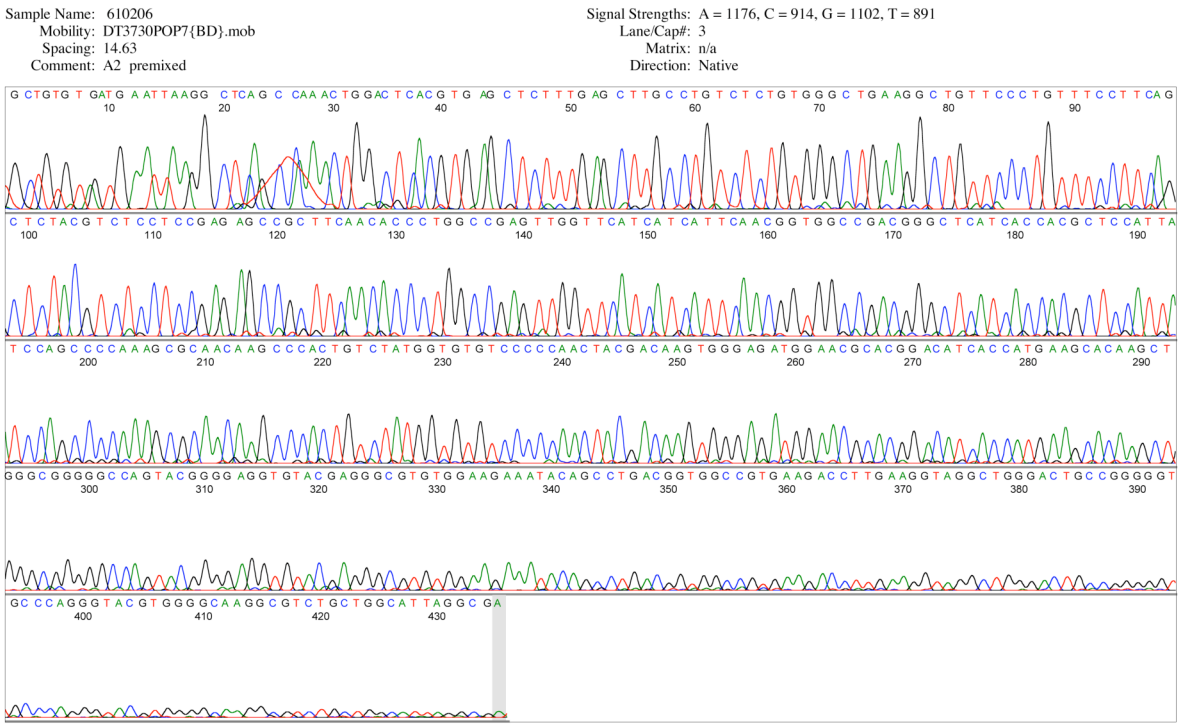

Figure 4.4. Example of the chromatogram file, showing the DNA sequence from exon 2 of patient no. 33 (courtesy of Roten T, InPheno, Basel)

No mutations of the *BCR-ABL* gene (on the two exons analyzed) could be detected (i.e. all patients received the code = 0). By contrast, various mutations were detected for the *KIT* gene: deletions, point mutations or mixed mutations: on exon 11 (code = 0), or alternately insertion in exon 9 (AY 502-503 duplication) or wild-type profile (code = 1).

Initially, this second pharmacodynamic exploration was conducted with the whole population (CML and GIST patients pooled). As in the standard exploration (§ 4.3.1), it revealed an inverse relationship between Dose, AUC and CL and therapeutic response ($p < 0.05$ in per-sample analysis, non-significant in per-patient analysis). It is important to note that this logistic regression analysis showed also that the response was significantly correlated with the mutation profile ($p < 0.05$ in per-sample and per-patient analysis), with patients presenting a resistance-related profile (i.e. *KIT* exon 9 mutation or *KIT* wild-type) being poor responders. The side effects analysis showed that Dose and AUC were still positively correlated with the amount of side effects when incorporating the mutation profile in the analysis ($p < 0.05$ in per-sample analysis, non-significant in per-patient analysis).

As in the standard PK-PD analysis, using the exposure estimates derived from the AGP model (i.e. *free* drug exposure) made the relationship between free AUC and response become positive. It was even significant when taking into account the pathology diagnosis ($p < 0.005$ in per-sample analysis, non-significant in per-patient analysis). The same was observed with CL_u,

with a negative relationship when incorporating the pathology diagnosis in the statistical model ($p < 0.05$ in per-sample analysis, non-significant in per-patient analysis), indicating that the greater is the free clearance value, the poorer is the response. Concerning the tolerability of the drug, free AUC remained positively correlated with the amount of side effects and CL_u showed still some association with tolerability scores ($p < 0.005$ in per-sample, and $p < 0.05$ per-patient analyses).

Finally, as it appeared that the mutation profile was highly correlated with the pathology diagnosis (individuals with resistant genetic profile were indeed only encountered in the GIST subpopulation), it was decided to also perform this last analysis focusing only on the GIST subpopulation. In this last per-patient analysis including the mutation profile, free AUC tended to be correlated with good response ($p < 0.1$) and CL_u proved to be significantly inversely correlated to response ($p < 0.05$). Of importance, CL_u appeared even as a better predictor of the response than the mutation profile (characterized only by a trend to influence response in this subpopulation; $p < 0.1$). These results are depicted in Figure 4.5, which presents the median free AUC and CL_u (with the associated box plot; generated by Stata®) corresponding to the 3 types of response (per-sample representation).

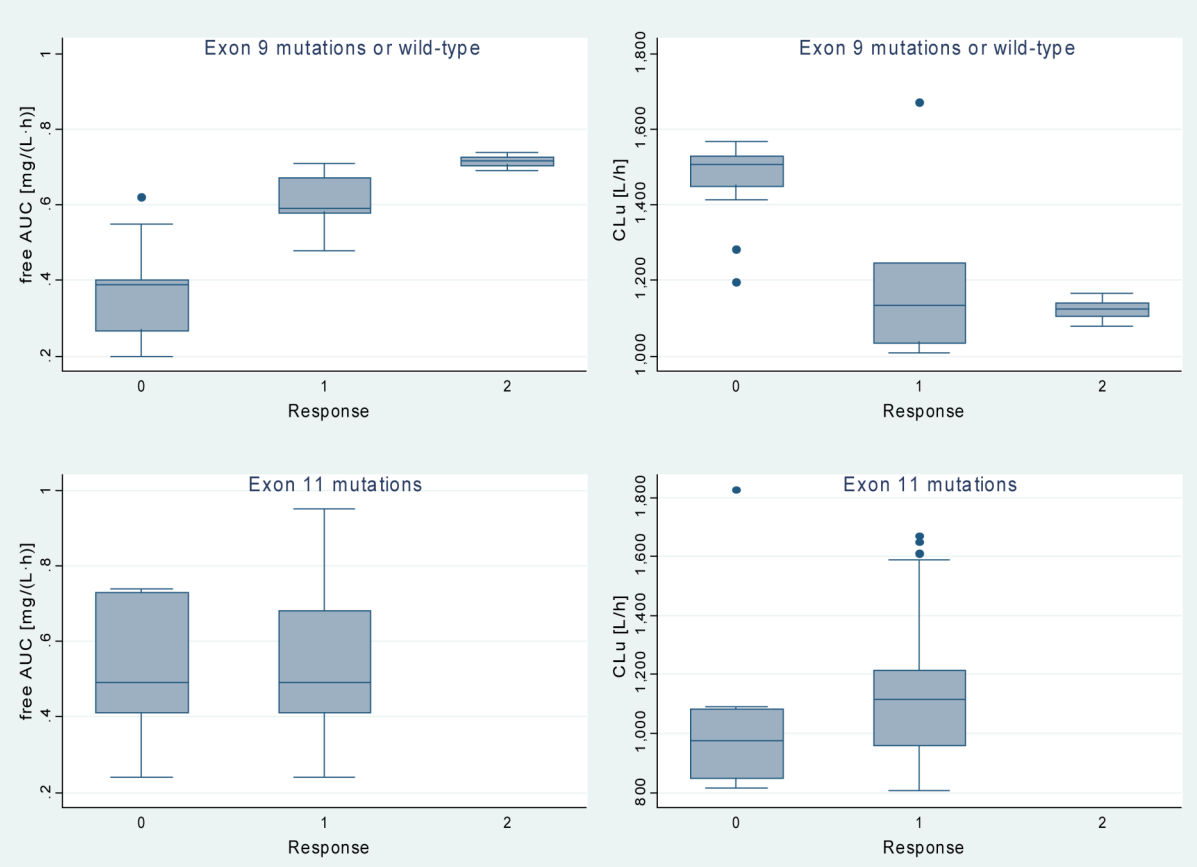

Figure 4.5. Box plot of the pharmacokinetic parameters according to response for the two main genotypic profiles of GIST patients (0 = progressive disease (n = 15 for exon 9/wt, 11 for exon 11), 1 = partial response/stable disease (n = 27 for exon 9/wt, 67 for exon 11), and 2 = complete response (n = 8 for exon 9/wt, 0 for exon 11))

4.4 Discussion

The preliminary standard PK-PD analysis shows that the occurrence of side effects is more frequent at higher imatinib exposure, indicating that monitoring imatinib plasma levels may therefore identify patients with excessive levels at risk of developing toxicity. Several cases (including a patient case at CHUV, described in detail in § 5.1) have indeed been reported in the literature where imatinib treatment had to be discontinued because of the occurrence of serious adverse events [14-16]. However, in only one case were plasma drug measurement and dose adjustment considered [16]. Conversely, several studies suggest that the administration of higher doses than the typical 400 mg daily regimen could improve the response in some patient subsets. A better response was indeed observed in accelerated and blast phases of CML with 600 mg daily [17], and a 800 mg daily regimen allowed a longer progression-free survival in GIST patients [18]. The inverse relationship we have observed between Dose (as well as AUC) and therapeutic response could thus be considered paradoxical. However, since this first study was only observational, we were in presence of good-responders receiving low doses of drug and bad-responders receiving high doses, without advantage. It must indeed be emphasized that the PK/PD relationships for a targeted agent such as imatinib may be obscured, or even confounded, by the somatic innate or acquired genotypic heterogeneity –with functional consequences, in term of susceptibility– of the intracellular pharmacological targets Bcr-Abl and c-Kit. In such a context, this issue has been addressed in the present study by also incorporating the imatinib target genotypes of CML and GIST patients into the statistical analysis.

The integration of the target mutation profile has therefore allowed refining our exploration of the PK-PD relationships in our population. However, among the CML patients for whom the principal *BCR-ABL* mutations were examined, none of them seemed to present any mutated *BCR-ABL* gene. A number of hypotheses could explain this observation. First, among the 20 CML patients, most of them (17 patients) were in chronic phase. It has been recently suggested –and demonstrated– that detection of mutant clones is not possible during the chronic phase of CML, but only during accelerated or blast phases [19]. Secondly, only three patients from our CML population were thus in accelerated or blast phase (and characterized by an intermediate or absent response). As it has been shown that about only 30% of such patients may present mutations [19,20], the very small number of patients included in the present study explains the low probability of detecting any mutations in our CML population. Moreover, the genotypic analysis of Bcr-Abl is generally done on RNA material, which was not possible in our

case (see above, in § 4.2.2.1). In fact, some patients of a recent observational study had reportedly to be excluded because of the poor quality of isolated RNA samples [21].

Alternately, the inclusion of the genetic profile of *KIT* into the statistical PK-PD analysis of the GIST subpopulation has allowed uncovering interesting relationships between free AUC and free CL, and clinical response. For example, Figure 4.5 suggests that patients with tumors harboring a protein c-Kit with a sensitive profile (resulting from exon 11 mutations of *KIT*) are exposed to concentrations that are already at the top of the concentration-response curve, whereas patients with a resistant profile (exon 9 mutations or wild-type *KIT*) are probably lying in the steep part of the curve, where a concentration-response relationship can be observed, and could probably clinically benefit from an adjustment in imatinib exposure. This study thus provides additional evidences of the existence of a clinical PK-PD relationship for imatinib and confirms the preliminary results of Peng *et al*, observed in a CML population [1]. Moreover, our results clearly show that the total plasma concentration of imatinib is a poor marker of its clinical effect. It is thus rather the free exposition (or free levels), either measured or calculated, that should ideally be taken into account for an eventual TDM program of imatinib.

Finally, it is worth noting that circulating imatinib does not account for all active drug, since approximately 10% of the dose is indeed metabolized by CYP3A4 into a N-desmethylated derivative (CGP74588) characterized by a similar *in vitro* potency [22]. As the half-life of elimination of this metabolite is about twice that of imatinib, it's accumulation after repeated dose intake is greater than the one of the parent compound (accumulation factor of about 2 to 3 *versus* 1.5 to 2 for imatinib, for once and twice daily intake, respectively; see also § 1.3.4). Practically, it appears that it represents less than 20% of the total imatinib in plasma at steady state [23]. Such an amount of active metabolite should not impact much, neither on PK, nor on PK-PD analysis. There would be however some interest to measure this compound in future studies.

In conclusion, our study was just exploratory and the results are based only on a very small population. The *p*-value obtained in our analysis should indeed be considered with caution, because of the limited number of observation. These results should however stimulate further stringent clinical studies in a larger patient population, aimed at confirming the relationships observed between imatinib pharmacokinetics –more precisely, imatinib free level–, and its efficacy and toxicity. By the way, a French group has just recently published a description of a LC-MS/MS method used for clinical monitoring of imatinib, to evaluate adherence to therapy, drug-drug interaction and PK-PD relationships [24]. In addition to the important variability

demonstrated and quantified in Chapter 3, the potential PK-PD relationship of imatinib represents another argument to evaluate further the potential benefit of a TDM program for this drug by a well-controlled clinical trial.

4.5 References

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Chapter 5 Clinical applications

This chapter is devoted to the presentation of two selected case reports of patients taking Glivec® and for whom clinicians requested imatinib blood measurement in our laboratory for helping them to optimize the treatment. Even though the clinical usefulness of imatinib TDM will require formal validation in a carefully controlled prospective randomized trial, these case reports provide examples of the potential applications –and clinical usefulness– of the monitoring of imatinib plasma levels in day-to-day clinical practice. These two case reports have been presented in abstract form at medical congresses [1-3], and one has been published in a peer-review international journal [4].

5.1 Severe pustular eruption associated with imatinib and voriconazole

5.1.1 Description of the case [1,4]

A 42-year-old Caucasian man had been followed for CML since 1998 and treated by Glivec® 400 mg qd since the beginning of 2002, increased to 400 mg bid on July 2002 (because of CML evolution into acute myeloid leukemia). In December 2002, an invasive pulmonary apergillosis (*Aspergillus fumigatus*) was diagnosed. A systemic treatment with fluconazole was started, relayed with oral voriconazole in January 2003. Twelve weeks after voriconazole initiation, this patient developed a confluent, infiltrated, papulopustular cutaneous eruption involving the face, the trunk, the arms and the hands, but sparing the folds, associated with high fever (Figure 5.1). The patient was followed by the Dermatology service at CHUV. Microbiological examinations were negative and the histological examination of a skin biopsy specimen showed a neutrophilic and eosinophilic infiltrate within the superficial dermis, without any sign of a Sweet's syndrome or CML specific cutaneous localization. The plasma levels of imatinib were measured using our HPLC-UV methods at several occasions between December 2003 and February 2004 (see for instance the chromatogram on Figure 5.2). These levels were markedly elevated during the simultaneous administration of voriconazole (between 3.5 to 4.1 μg/ml, about 14 h after administration), when compared with the predicted levels of about 2 μg/ml extrapolated from a previously published pharmacokinetic study (determined at steady state for a 600 mg daily dose) [5]. The skin condition improved within three weeks of stopping imatinib and

voriconazole, and administration of 30 mg daily oral prednisone. Voriconazole was reintroduced one month later without any recurrence of the eruption.

Figure 5.1. Papulopustular nonfollicular eruption tending to colasescence to form plaques with irregular mamillated surface, concerning the face (a), the trunk, the arms and the hands (b) (Copyright 2005 S. Karger AG, Basel. All rights reserved. Reproduced with permission from Dermatology, 211, Gambillara E et al, Severe pustular eruption associated with imatinib and voriconazole in a patient with chronic myeloid leukemia, 363-5)

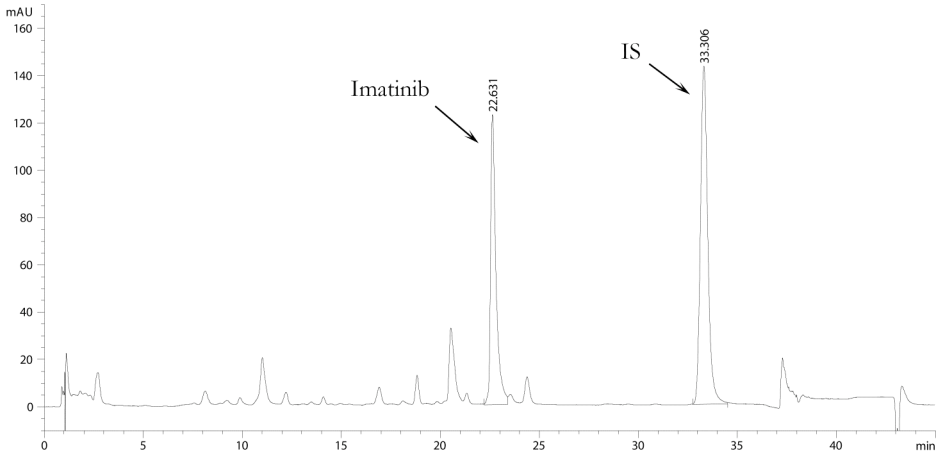

Figure 5.2. Plasma chromatogram of the CML-patient presented in this case report, obtained 14 h 30 min after imatinib intake (400 mg, bid)

5.1.2 Discussion [1,4]

Adverse cutaneous reactions induced by imatinib are frequent, and usually observed in 7 to 21% of patients, with 5% of such reactions being severe or life threatening [6,7]. In a recent study, 48 out of 54 (88%) patients with Philadelphia chromosome-positive leukemias treated by imatinib experienced at least one cutaneous reaction. Maculopapular rash, edema and pruritus

were the most common adverse effects. The rash prevalence rate was significantly higher in females, as generally reported with other cutaneous drug reactions [8].

In patients treated with imatinib, rashes other than non-specific maculopapular eruption have also been observed [9,10]. The case reported here consists of an unusual and severe skin side effect following imatinib presenting as a papulopustular eruption. Two similar cases have already been described [11], and classified under the denomination of AGEP (acute generalized exanthematous pustulosis). However, these two patients, as well as ours, actually suffered conditions not exactly corresponding to this dermatosis. Indeed, the patients presented cutaneous lesions differing from those of AGEP. This notably because they appeared more than 3 months after the beginning of the treatment, and because the papulo-pustular eruptions concerned mainly the face and the trunk, and spared the folds.

The incidence and severity of imatinib adverse cutaneous reactions appear to be dose dependent [7,12,13], with mild reaction at low to intermediate doses (200-600 mg daily), and severe eruptions at higher doses (600-1000 mg daily) [13]. This pattern suggests that skin reactions due to imatinib are related to a pharmacological effect of imatinib rather than to a hypersensitivity process. This in turn indirectly supports our observation of severe cutaneous toxicity associated with high plasma levels of imatinib in our patient.

As already mentioned, drugs inhibiting CYP3A4 activity, such as voriconazole and fluconazole [14], decrease the metabolism of imatinib, and this should results in increased drug plasma levels [15], as seen in our case. The very high levels of plasma imatinib that were measured probably reflect pharmacokinetic interactions, mediated by the inhibition of imatinib metabolism by voriconazole. The prolonged exposure to high levels of imatinib could thus have led to the development of the skin eruption. However, no plasma level values of imatinib *before* the administration of the two antifungal drugs were available for our patient, and this allows us only to suggest such a hypothesis.

5.2 Dose adjustment of imatinib based on plasma level measurement

5.2.1 Description of the case [2,3]

A 26-year-old man was diagnosed as having CML in chronic phase at low risk (according to Hasford Score). He was taken in charge by the Inselspital in Bern (University hospital) and placed on hydroxyurea. After hematological remission, he was treated with interferon-α at 8 MIU/day. The interferon-alpha treatment was stopped because of hematological side effects. Administration of imatinib was started at 400 mg daily, and after 9 months cytogenetic analysis showed Philadelphia (Ph) chromosome positivity in approximately 70% of metaphases, and no decrease of *BCR-ABL* transcripts was noted by quantitative PCR. Because of a body weight of 110 kg, plasma level measurements of imatinib were performed in our laboratory at PCL, and revealed that the plasma level (i.e. 0.4 µg/ml, 25 h 30 min after dose intake) was half of the expected values based on the pharmacokinetic data reported at steady state (see previous case) [5]. The daily intake of imatinib was therefore increased to 600 mg administered in two doses. After 3 and 6 months, repeated cytogenetic analysis showed a major cytogenetic reduction (7% of Ph+ metaphases in conventional method and 1% by FISH analysis[1]), as well as almost a 3 log reduction of *BCR-ABL* transcripts. The CML evolution in this patient is illustrated in Figure 5.3.

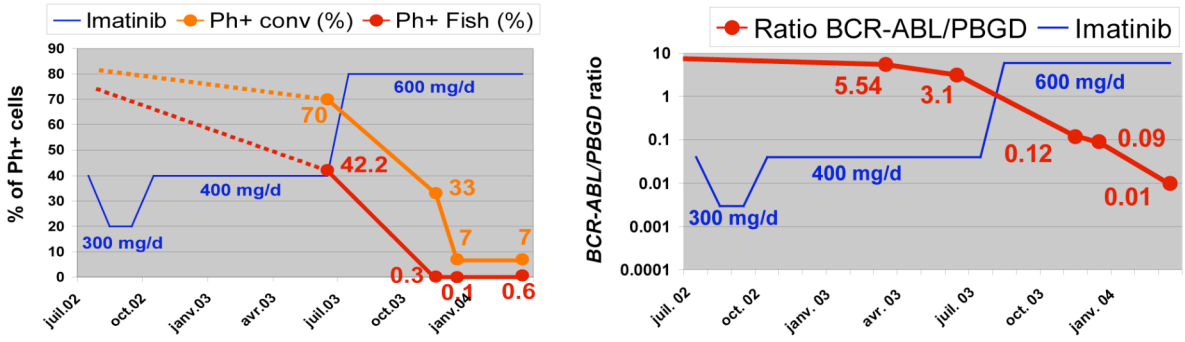

Figure 5.3. Cytogenic course (left; conv = conventional) and quantitative BCR-ABL-transcripts course (right; PBGD = reference gene) (courtesy of Heizmann M, with modifications)

[1] FISH (Fluorescent *in situ* hybridization) is a cytogenetic technique that can be used to detect and localize specific DNA sequences on chromosomes. It uses probes that become fluorescent when binding to parts of the chromosome showing a high degree of sequence similarity.

5.2.2 Discussion [2,3]

As stated in Chapter 1, imatinib is metabolized by CYP3A4 known to present a high interindividual variability in expression and activity, and is likely to be inhibited or induced by numerous dietary and environmental compounds. This suggests that exposure to imatinib is highly variable and may be even suboptimal for some individuals. The case described here presents a dose adjustment of imatinib based on plasma level measurements in a patient with CML, which resulted in an improved cytogenetic response. However, the exact reason for the low initial imatinib level is not known. Neither CYP3A4 phenotyping nor CYP3A4 genotyping, nor blood AGP levels measurement (also found to influence imatinib clearance; see Chapter 3) have been performed in this patient.

5.3 General discussion

The two described case reports are typical clinical examples regularly encountered during the course of the present study. Very often indeed, practitioners from all Switzerland, facing either an absence of therapeutic response or the appearance of serious side effects in their patients on imatinib, referred to us for imatinib blood measurements, to verify whether the patients' exposure to the drug was adequate. In fact, since the beginning of the present study (in Summer 2002) until March 1, 2006, as much as 90 requests for imatinib plasma levels measurements –for a total of 38 patients from all over Switzerland– have been addressed to our laboratory, most of whom outside the study protocol.

Despite the fact that no significant influences of CYP3A4 inhibitors on imatinib pharmacokinetics could be statistically demonstrated in our patients' population (see § 3.3), the first presented case certainly suggests that the addition of strong CYP3A4 inhibitors such as voriconazole to the imatinib treatment has to be considered with caution. Cutaneous reaction to imatinib may indeed not be dose-related, but concentration-related instead. As far as the second case is concerned, it has allowed demonstrating that the measurement of imatinib plasma level can be useful to ascertain plasma exposure and for the adjustment of imatinib dosage, and has resulted in an improved cytogenetic response. Figure 5.4 graphically represents the imatinib concentrations of the two cases (for a normalized 400 mg daily intake) compared to the whole patient population with the prediction intervals defined in Chapter 3 (see Figure 3.6). This Figure clearly underscores first, how variable can be imatinib plasma concentration in a patient population for a same Glivec® dose, and secondly, that the imatinib plasma levels found in the above-mentioned case reports no. 1 and 2, lie in the upper, and lower range of the predicted

concentration interval, respectively. However, it is noteworthy to emphasize that no therapeutic interval for imatinib has formally been established yet. This could be determined only after the initiation of comprehensive PK-PD studies, also taking into account the genetic heterogeneity of imatinib pharmacological targets in patients (see Chapter 4 for further details).

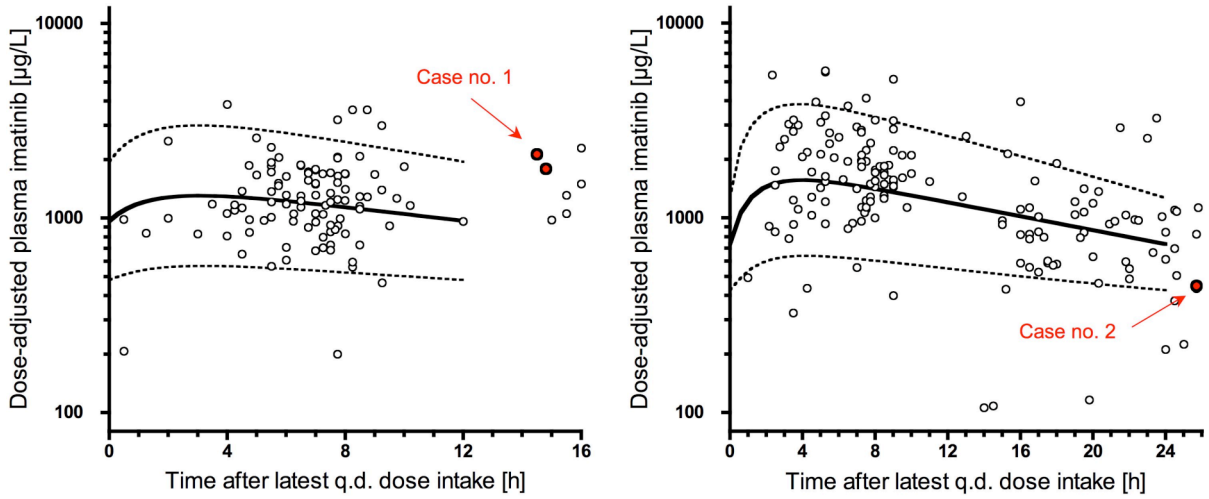

Figure 5.4. Imatinib plasma concentrations observed in patients receiving imatinib, along with the average population prediction (solid line) and 90% prediction interval (dashed lines). The values are adjusted to 400 mg daily: 200 mg bid and 400 mg QD on the left and right part, respectively. The patient concentrations of case no. 1 and 2 are shown on the left and right part, respectively

5.4 A first step toward TDM of imatinib in clinical practice

As exemplified by the two above-mentioned cases, information on imatinib plasma exposure can be important to help clinicians in the optimal follow-up of their patients. More generally, routine monitoring of imatinib plasma levels has been found useful to identify individuals with imatinib concentration exposure grossly departing form population average, in the presence of toxicity or in the absence of clinical response.

Since a growing number of physicians throughout Switzerland were asking for information on imatinib blood concentrations in their patients (see above), a user friendly graphical plot of the drug concentration over time after drug intake has been developed. Based on the NONMEM analysis performed in our study population (see Chapter 3), we have being able to establish percentile curves for the various imatinib regimens (using a simulation with the Bateman model; see § 3.3.1.1 for further details). Figure 5.5 shows one example of such a percentile curve, which is now routinely provided to practitioners along with the TDM interpretation report. The full report of this example can be found in Appendix 5.1. The imatinib plasma levels measurements shown in this Figure are those from one patient whose imatinib plasma exposure was periodically

ascertained during his treatment between 2002 and 2005. This was done in accordance to the physician's request and patient's own scientific interest. Noteworthy, these data illustrate the low intraindividual variability in imatinib exposure in this responding patient, mostly comprised in the upper percentiles range.

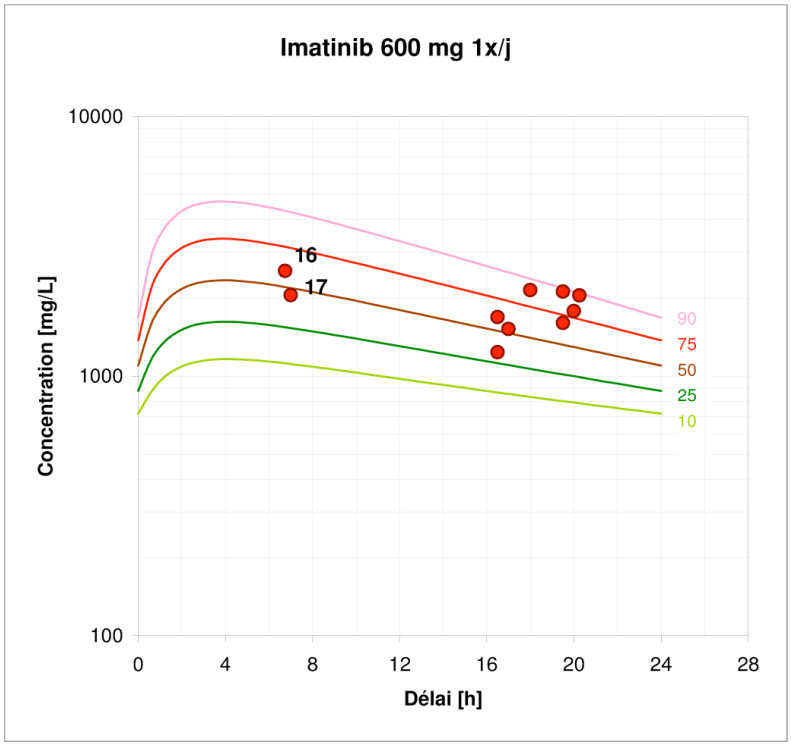

Figure 5.5. Percentile curves of imatinib for the imatinib 600 mg daily regimen, along with concentrations measured routinely in one patient during the period 2002-2005

However, it is important to note that such concentration interpretation does not represent a formal TDM approach yet. Our interpretations are only taking into account the pharmacokinetic variability of the drug, but are not formally based on any therapeutic interval. Additional pharmacokinetic-pharmacodynamic studies on imatinib are thus needed before systematically recommending dose adjustments based on plasma levels, in patients lacking adequate clinical responses. As already stated, gathering further information to better predict the potential effectiveness of a TDM strategy for imatinib is thus necessary.

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Chapter 6 Intracellular disposition

In connection with the main clinical research project, we were also interested in studying imatinib disposition at the cellular level using *in vitro* cell cultures, and in determining the influence of *MDR1* gene expression and specific gene silencing (using a siRNA approach), on the differential cellular handling of imatinib. Efflux as well as uptake drug transporters are indeed increasingly recognized as key determinants of drug disposition and clinical response (see also § 1.4.3) [1].

Complementary to the studies on the effect of the efflux transporter P-gp expression and silencing, this chapter will also present some preliminary experimental data on the presumed influence of the uptake transporter hOCT1 (described in § 1.4.3) on imatinib intracellular disposition.

Even tough these *in vitro* experiments would first appear only partially related to our main research objective aiming at evaluating the potential benefit of a TDM program for imatinib, such studies are however essential to gain a more in depth appreciation of the tissular and cellular disposition of imatinib (or of any other new anticancer drugs) in the body. Since several drug efflux/influx transporters involved in imatinib cellular disposition are the subject of various polymorphisms, with consequences in terms of expression and function [1], gaining additional knowledge in this field could help us designing randomized TDM trials that also take adequately into account the patients' genetic variability associated to these transporters.

6.1 Functional consequence of *MDR1* expression on imatinib intracellular concentrations

In an article published in 2003, Mahon *et al* [2] have demonstrated that *MDR1* gene overexpression can confer resistance to imatinib in leukemia cell lines. Beside *MDR1* overexpression, several other cellular mechanisms of resistance to imatinib have also been identified (see § 1.4 for further details) [3-5]. As already mentioned, the generally accepted functional consequence of *MDR1* gene expression is the reduction of intracellular drug accumulation through P-gp-mediated efflux, thus hampering the achievement of effective drug levels at the target site. Mahon *et al* [2], have shown that *MDR1* gene overexpression results in resistance to imatinib, however without assessing the functional consequence of *MDR1* expression on imatinib intracellular availability. As a complement to their work, we have

performed *in vitro* cellular experiments that provide a first direct evidence of the marked impact of P-gp expression on imatinib intracellular concentrations.

The cell culture and incubation experiments described below were performed in the Laboratory of Clinical Chemistry of our hospital with the technical help of Dr Hugues Henry and Sara Colombo (PhD student).

6.1.1 Materials and methods

6.1.1.1 Imatinib incubation with cell culture

Homologous *MDR1*+ and *MDR1*- LLC-PK1 cells (i.e. transfected porcine kidney epithelial cells, a recognized model for assessing drug efflux transporter activity [6]) were obtained from Schinkel [7]. Cells were incubated for 18 h at 37°C, in 2 ml growth medium containing 10% fetal calf serum in the presence of imatinib at the clinically relevant concentrations of 0.5, 1.5 and 3.0 μg/ml. All concentrations were tested in duplicates. Following incubation, a 300-μl aliquot was removed to determine the extracellular concentration. The excess supernatant was discarded, and the resulting adherent cell cultures were washed three times with an ice-cold phosphate buffered solution (PBS). Each cell culture was then extracted with 1 ml MeOH/H_2O 60 : 40 on a planar shaker (Unimax 2010, Heidolph, Kelheim, Germany) by gentle agitation at 100 rpm for 3 h. The methanolic solutions were collected in Eppendorf vials and centrifuged at 20'000 g (14'000 rpm) for 10 min at RT (Benchtop Universal 16R, Hettich, Bäch, Switzerland). The supernatant was finally evaporated to dryness with a nitrogen flux and the residues were reconstituted in 200 μl MeOH/H_2O 60 : 40 prior to analysis using a slight adaptation of the HPLC-UV method described in § 2.1.

6.1.1.2 Imatinib measurement by LC-UV in extra- and intracellular extracts

Extracellular (i.e. cell culture media) and intracellular (i.e. methanolic) extracts were thus measured using the LC-UV method previously developed. However, for this *in vitro* study, calibrations curves were prepared either in the culture medium diluted 2 : 3 with MeOH/H_2O 60 : 40 for extracellular measurement, or directly in a MeOH/H_2O 60 : 40 mixture for intracellular measurement of imatinib. The 200-μl extracts obtained during the *in vitro* study were diluted 2 : 3 with MeOH/H_2O 60 : 40, followed by a 10-min centrifugation (Benchtop Universal 16R, Hettich, Bäch, Switzerland), prior to injection. The established calibration curves were characterized by an excellent regression coefficient (r^2 = 0.997).

6.1.1.3 Total protein measurement

The concentrations determined in the 24 extracts (12 intra- and 12 extracellular) were normalized on total proteins measurements, used as surrogates of cell counts. This determination of protein was carried out by solubilizing the adherent cell cultures with a 400-µl solution containing 8 M urea and 1% sodium dodecylsulfate. These solutions were placed in the Eppendorf tubes previously used to collect the methanolic extracts, so that the totality of proteins (i.e. including those possibly carried during the methanolic extraction) could be retrieved. The solutions were then assayed for total protein using the bicinchonic acid (BCA) assay [8].

This assay was performed using the "BCA protein assay" reagent kit (Pierce Chemical, Rockford, USA). It is a mere spectrophotometric dosage (with calibration) after the reaction of the proteins with the biuret reagent in a 96-well plate. The classical biuret reaction consists in the formation of a colored complex formed between the peptide bonds of proteins and the copper cations Cu^{2+} of the reagent [9].

6.1.1.4 Transporters inhibition by rotenone

The same experiment was repeated by adding rotenone[1] to the culture media, aiming at inhibiting the ATP-dependent activity of P-gp in cells (following Jones *et al* procedure [10]). This experiment aimed at examining whether the intracellular accumulation obtained with *MDR1*- cells would be similar to *MDR1*+ cells inhibited by rotenone.

6.1.2 Results

6.1.2.1 Initial experiment

The results given in Table 6.1 highlight the striking differences (up to 24-fold) in intracellular concentration of imatinib as a function of the cell ability to express P-gp. These results have been published in 2003 in *Blood* [11]. Figure 6.1 shows the comparison of intracellular accumulations of imatinib, normalized according to proteins content, used as a surrogate for cell counts.

[1] Rotenone is a naturally occurring compound isolated from the roots of several tropical plants (e.g. *Derris elliptica* Benth.), used for more than 2'000 years in equatorial countries as fish poisons. It interferes with NADH dehydrogenase, preventing NADH to act as a proton and electron donor in the aerobic cell respiratory chain. Rotenone thus inhibits any active (i.e. requiring ATP energy) transport in the cell.

Table 6.1. Concentration of imatinib in *MDR1+* and *MDR1-* cells at various imatinib incubation levels

Genotype	Incubation medium [ng/ml]	Intracell. [ng/μg prot]	Extracell. [ng/μg prot]	Intra/extracell. ratio
MDR1+	500	0.07 ± 0.006	2.86 ± 0.481	0.023
	1500	0.10 ± 0.002	5.70 ± 0.687	0.017
	3000	0.26 ± 0.031	12.70 ± 0.322	0.020
MDR1-	500	0.24 ± 0.004	3.04 ± 0.143	0.079
	1500	1.15 ± 0.190	5.92 ± 0.326	0.195
	3000	6.14 ± 0.432	11.17 ± 0.384	0.550

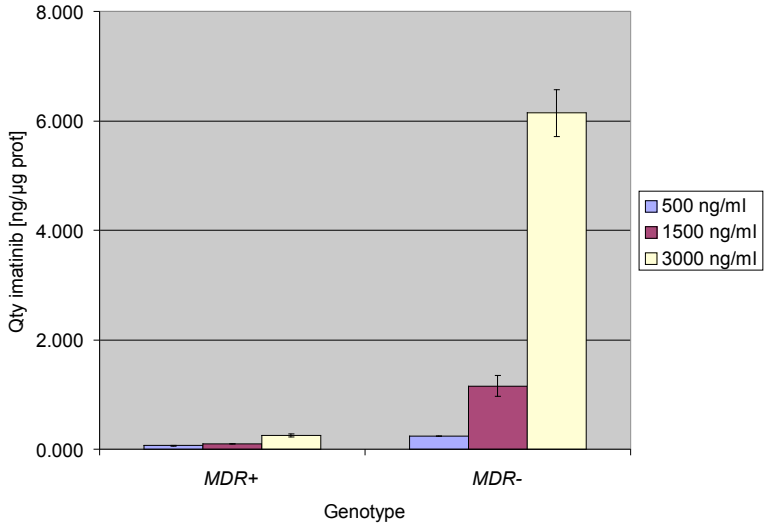

Figure 6.1. Intracellular accumulation of imatinib at incubation concentrations of 500, 1500 and 3000 ng/ml (= μg/l)

Our experiment provides a direct evidence of the influence of P-gp on imatinib intracellular availability. Imatinib is very efficiently expelled from *MDR1+* cells at all tested concentrations. Intracellular concentrations and transmembrane ratios are significantly affected by genotype ($p < 0.0001$) and applied concentration ($p < 0.0001$, two-way ANOVA).

6.1.2.2 Drug transporters inhibition by rotenone

The results of the transport inhibition with rotenone (Table 6.2 and Figure 6.2) essentially reveal almost no difference in imatinib cellular exposure between *MDR1-* and *MDR1+* cells. In fact, paradoxically, the value of the intra/extracellular ratio in the presence of rotenone was in both cell genotypes very low, about 0.014 (Table 6.2), being essentially the same as that measured in *MDR1+* cells in the first experiment (i.e. without P-gp inhibition; Table 6.1).

Table 6.2. Concentration of imatinib in *MDR1+* and *MDR1-* cells at various imatinib incubation levels in the presence of rotenone

Genotype	Incubation medium [ng/ml]	Intracell. [ng/µg prot]	Extracell. [ng/µg prot]	Intra/extracell. ratio
MDR1+	500	0.12 ± 0.001	9.32 ± 0.481	0.013
	1500	0.37 ± 0.065	24.92 ± 0.687	0.015
	3000	0.90 ± 0.235	78.47 ± 0.322	0.012
MDR1-	500	0.25 ± 0.107	17.36 ± 0.143	0.014
	1500	0.44 ± 0.001	29.77 ± 0.326	0.015
	3000	1.18 ± 0.082	70.13 ± 0.384	0.017

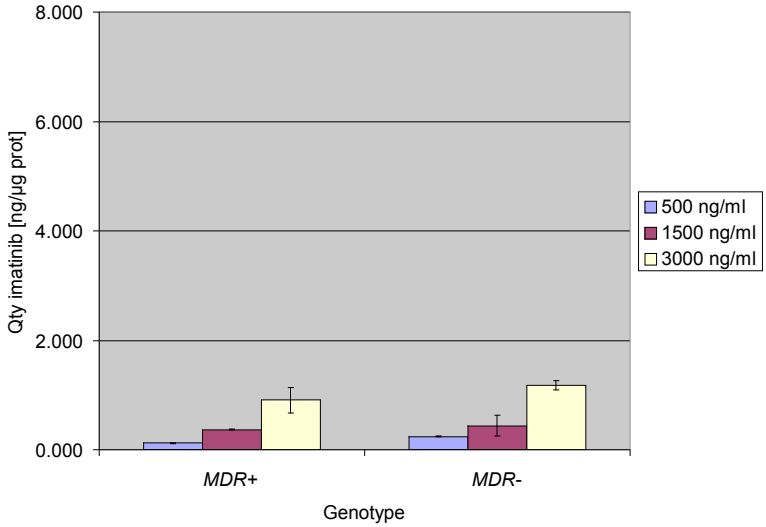

Figure 6.2. Intracellular accumulation of imatinib at incubation concentrations of 500, 1500 and 3000 ng/ml (= µg/l), in the presence of rotenone

6.1.3 Discussion and conclusion

Table 6.1 shows that in *MDR1+* cells, the intra/extracellular concentration ratio remains constant, indicating that imatinib P-gp-mediated efflux is never saturated at all tested concentrations. By contrast, the higher ratio observed at low imatinib level in *MDR1-* cells further increases at higher levels, and this suggests the existence of subsidiary, less efficient and saturable transport mechanisms. It is however important to note that, as the volume of Schinkel cells was not known in this experiment, it was necessary to normalize on total protein amount. Therefore, the absolute value of the intra/extracellular ratio has no physiological relevance (contrary to a concentration ratio). Instead, relative, rather than absolute ratio values were of interest in this comparative study.

This experiment formally confirms that imatinib interaction with P-gp [12] has important functional consequences, and that imatinib efflux from cancer cells by the drug transporter P-gp should be seriously considered among the mechanisms of imatinib resistance. Despite the still

debated role of P-gp as a resistance factor to imatinib [13], our results supports the initial observations by Mahon *et al* [2].

Moreover, our modulation experiments with rotenone resulted paradoxically in very low intracellular concentrations of imatinib in both types of cells. The contrary (i.e. higher cellular levels) would have been expected from the sole inhibition of active *efflux* transport system such as P-gp. One explanation for this observation could be the simultaneous inhibition of active *uptake* transporters system implied in imatinib influx inside cells, in addition to the inhibition of efflux transporters. This simultaneous inhibition of active efflux and influx transport systems by rotenone could thus result in a net intra/extracellular equilibrium due to the sole passive diffusion of imatinib through the cell membranes. This is in line with the recent demonstration that imatinib undergoes active transport *inside* leukemia cells by the human cationic transporter hOCT1 [14].

Overall, our experiments support the hypothesis that the clinical activity of imatinib may thus be significantly hampered in P-gp expressing cells, and this seems to be particularly the case of CML cells in blast phase [15,16]. Moreover, differences in activity of imatinib uptake transporters seem also to have some clinical consequences, as reported in a recent study whereby hOCT1 expression levels were found significantly higher in responders than in non-responders CML patients [17]. In line with the previous discussion in § 3.4, these findings should stimulate further research to evaluate the effect of addition of P-gp inhibitors and/or hOCT1 modulators to imatinib for the treatment of CML blastic crisis.

6.2 Resistance reversal by siRNA *MDR1* in CML cells is caused by an increase of imatinib intracellular disposition

The cell culture and incubation experiments described in the following paragraphs were performed in the Laboratory of Tumor Biology and Angiogenesis of the Medical University of Innsbruck (Austria) by Holger Rumpold *et al.* The intracellular imatinib measurement by LC-MS/MS was carried out in our laboratory in Lausanne.

6.2.1 Theoretical principles

Small interfering RNA (siRNA), are a class of 20-25-nucleotide long RNA molecules whose importance in cellular biology is being increasingly recognized since their recent discovery. They are mainly implicated in the RNA interference pathway (RNAi), whereby the siRNA interferes

with the expression of the specific gene that shares a homologous sequence with the RNA [18,19]. It is a naturally occurring mechanism of defense and a regulating process, and many hopes and research efforts are put in this approach to transform this gene expression modulation approach in a viable therapeutic concept.

In the context of *MDR1* gene modulation, Rumpold *et al* have shown that RNAi-mediated knockdown of P-gp using a transposon[2]-based vector system durably restores imatinib sensitivity in imatinib-resistant CML cell lines K562/Dox [20]. Decreasing, if not abolishing, P-gp expression through the silencing of *MDR1* gene thus represents an attractive strategy to revert drug resistance, and the use of the SB-based RNAi system seems to be a powerful tool for that purpose. This system consists in a SB-based[3] vector [21], into which a cDNA oligonucleotide coding for a siRNA was inserted. In this case, the siRNA was targeted against the *MDR1* gene [20].

The specific aim of our experiment was therefore to ascertain whether the resistance reversal observed in the above mentioned resistant cell lines subjected to *MDR1* siRNA-mediated silencing was associated with –and could therefore be explained by– an increase of imatinib intracellular disposition.

6.2.2 Materials and methods

6.2.2.1 Imatinib incubation with cell cultures

P-gp overexpressing K562/Dox cells were obtained by long-term culture in the presence of doxorubicin by Marie JP at INSERM (University of Paris, France). K562 cells are an erythroleukemia cell line derived from a CML patient in blast crisis. These cells were cultured in Austria by Rumpold *et al* in RPMI 1640 medium containing 2% glutamine, penicillin, streptomycin (Invitrogen, Lofer, Austria), 10% fetal calf serum (FCS), and 1 nM doxorubicin. Half of them were then nucleofected with the siRNA transposon-based vector in order to obtain one K562/Dox-si*MDR1* cell line and one control cell line K562/Dox-si∅. The cells were propagated and stained for P-gp by FACS[4] to check for *MDR1* expression, and were then seeded into 6-well plates (900'000 cells/well). The two cell lines were incubated with 0 (simplicate),

[2] Transposons (or "jumping genes") are sequences of DNA that can move around to different positions within the genome of a single cell, a process called transposition. In the process, they can cause mutations and change the amount of DNA in the genome.
[3] SB stands for "Sleeping Beauty" (a classic fairy tail named "La Belle au Bois Dormant") in French, which in fact gave its name to a synthetic transposon derived from the genome of some fish species. This element shows efficient transposition in cells of a wide range of vertebrates, including humans.
[4] FACS (Fluorescent-activated cell sorting) is a tool to study and purify cells, based upon specific light scattering and fluorescent characteristics of each cell.

1 (duplicates), 3 (duplicates), 6 (simplicate) μM imatinib for 2 h. These concentrations correspond to 0.5, 1.5, 3.0 μg/ml (i.e. 500, 1500 and 3000 ng/ml) of imatinib, which are typical concentrations occurring in patients plasma samples (see Chapter 3). After incubation, cells were centrifuged and supernatant collected and frozen at -20°C. The resulting cell pellets were washed 3 times in cold HBSS buffer and the pellets frozen at -20°C. All samples (12 pellets and 12 supernatants) were shipped on dry ice to our laboratory in Lausanne for the determination of imatinib levels in cells and incubation media.

6.2.2.2 Imatinib measurement by LC-MS/MS in extra- and intracellular extracts

The supernatants and cellular pellets extracts were analyzed by LC-MS/MS according to the method described on § 2.2.2.2, but using a new system: a TSQ Quantum Discovery Ion Max tandem triple-stage quadripole (Thermo Finnigan, San Jose, USA) coupled to a Rheos 2200 Advanced Chromatography Platform (Flux Instruments, Basel, Switzerland)[5]. The supernatants were diluted with the RPMI medium 1/5 prior to analysis, along with calibration standards prepared in the same RPMI medium at concentrations of 0.001, 0.01, 0.05, 0.1, 0.5 and 1 μg/ml of imatinib. A 1-ml volume of imatinib-d8 0.2 μg/ml (internal standard) in ACN was then added to the diluted supernatant and the resulting solution was finally diluted 1/2 with "Buffer A" (= 10 mM NH_4Ac and 1% acetic acid, which corresponds to the aqueous mobile phase used for the chromatographic elution).

Cell pellets were extracted with 1-ml MeOH/H_2O 50 : 50 by vortex-mixing and ultrasonication for 15 min. A 1 ml-volume of imatinib-d8 0.2 μg/ml in ACN was then added to the cellular extract suspension, which was vortexed again and sonicated for 5 additional min. The mix was finally centrifuged and the resulting supernatant diluted 1/2 with the above-mentioned "Buffer A", which gave the analytical sample for the LC-MS/MS assay. The calibration samples were prepared in MeOH/H_2O 50 : 50 (levels: 0.001, 0.01, 0.05, 0.1, 0.5 and 1 μg/ml). The intracellular concentrations of imatinib were calculated assuming an intracellular volume of 0.4 pl, and a cell number of 900'000 in each well.

6.2.3 Results

The results given in Table 6.3 highlight the striking differences (between 4- and 9-fold) in intracellular concentration of imatinib as a result of *MDR1* gene expression knockdown. Figure 6.3 shows a comparison of intracellular accumulations of imatinib between K562/Dox

[5] Analysis carried out with the help of Alexandre Béguin (one of our laboratory technician).

cells expressing P-gp and K562/Dox cells with inhibition of P-gp expression via siRNA *MDR1* silencing.

Table 6.3. Concentration of imatinib in P-gp+ and P-gp- (RNAi knocked down) cells at various imatinib incubation levels

Phenotype	Incubation medium [μg/ml]	Intracell. [μg/ml]	Extracell. [μg/ml]	Intra/extracell. ratio
P-gp+ (siØ)	0.5	18.1 ± 3.93	0.7 ± 0.01	27.2
	1.5	69.4	2.0 ± 0.28	34.7
	3.0	206.7	3.9	52.5
P-gp- (siRNA)	0.5	206.9	0.7 ± 0.16	301.0
	1.5	433.6 ± 23.96	1.7 ± 0.14	250.7
	3.0	778.6	3.4	230.5

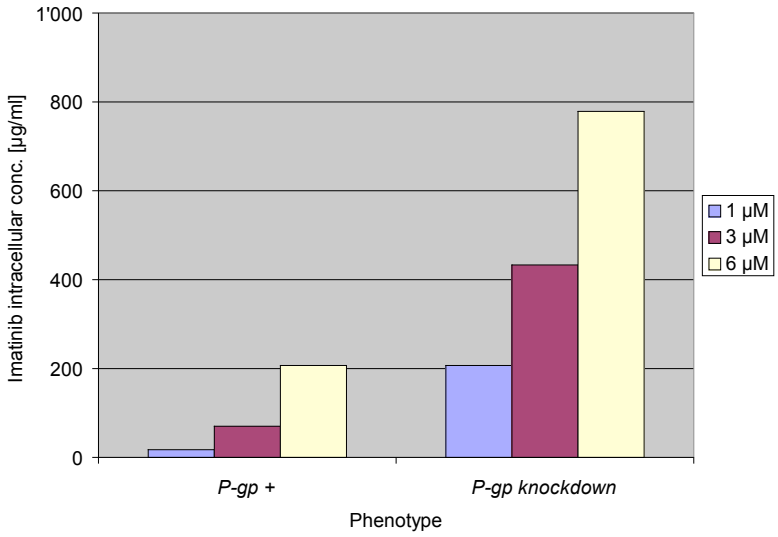

Figure 6.3. Intracellular accumulation of imatinib at incubation concentrations of 1, 3 and 6 μM (i.e. 0.5, 1.5 and 3 μg/ml), according to the cell phenotype after MDR1 gene expression silencing

Using the si-RNA approach, this experiment provides a first direct evidence of the influence of the *specific* P-gp inhibition on imatinib intracellular availability. Imatinib accumulates at a much higher extent in CML cells that have been knocked down for P-gp expression. Intra/extracellular ratio is significantly affected by phenotype ($p < 0.05$; two-way ANOVA), but not by applied concentration in the incubation (extracellular) medium.

6.2.4 Discussion and conclusion

In complement to the initial experiment of Rumpold *et al* [20], our analysis formally demonstrates that the restoration of imatinib sensitivity observed in the imatinib-resistant CML cell lines K562/Dox subjected to *MDR1* gene silencing is indeed the result of an increase in

imatinib intracellular disposition. In the P-gp knockdown cells, only the uptake process seems to occur, resulting in a marked imatinib cellular accumulation, as shown in the right-hand histograms of Figure 6.3.

However, the values of intra/extracellular ratios determined in the P-gp+ cells in these *in vitro* cellular experiments (IC/EC = about 40) are not of the same order of magnitude as the value previously determined *in vivo* in our clinical pharmacokinetics study with patients (8-fold factor, see § 3.3.4), or reported in a previous *in vitro* study (5-fold factor in leukemia cell cultures incubated with imatinib and human plasma [22]). The accumulation of imatinib was shown to be much higher in our experiment. The difference in the measured IC/EC ratio could probably be explained to some extend because these studies have been realized with different cell types (i.e K562, PBMCs, etc), and under different experimental conditions (*in vivo*, *in vitro*, in the presence/absence of proteins in the incubation medium). In the previously reported *in vitro* and *in vivo* experiments, the total imatinib concentrations were of the same magnitude. However, these experiments were carried out in the presence of human plasma proteins –notably AGP– in the extracellular environment, onto which imatinib is heavily bound (i.e up to 95% [23]; see also § 3.4). This indicates that the remaining free imatinib concentration available for interacting and stacking onto cellular lipid bilayers (and thus likely to be transported by P-gp/hOCT1) is much lower. As demonstrated in Chapter 3, the free imatinib concentration is indeed almost 50- to 100-fold lower than the total concentration. Conversely, in our *in vitro* study, only a small amount of non-human proteins (10% fetal calf serum) was added into the incubation medium. The extracellular concentrations of imatinib, which cells were actually exposed to, were accordingly substantially higher than in the previous *in vitro* experiment [22]. It is therefore probable that the free imatinib concentration gradient was much higher in our extracellular environment than in the previous studies. The cell-associated amount of imatinib measured in our experiment, and considered as an intracellular concentration, can thus not be formally compared to the previous results. However, as previously stated, the relative (rather than absolute) values of IC/EC ratios determined in P-gp- and P-gp+ cells were of interest in our experimental design. Consequently, the principal information that emerges from our measurements is that the reversal of imatinib resistance upon *MDR1* gene silencing observed by Rumpold *et al* [20] is indeed associated with a significant increase of imatinib accumulation into cells.

Such findings confirm thus the possible usefulness of using RNAi techniques to overcome imatinib resistance *in vitro*. It is certainly an argument to further evaluate such approach in animal models. More generally, because of its ultimate gene specificity, the overall RNAi strategy is very

attractive and the future years will probably be determinant for the evolution of this novel concept. As previously stated, many hopes and research efforts are put into this RNAi strategy approach to transform it into a viable therapeutic concept.

6.3 Development of cellular models for further *in vitro* studies of drug transport modulation

6.3.1 Introduction

As efflux and uptake drug transporters have been found to be essential determinants of drug disposition and clinical response [1,14], it is particularly relevant to explore in basic *in vitro* experiments the various ways to modulate these transport systems in order to possibly improve drug efficacy and/or to overcome resistance to treatment. In that perspective, the previously reported method with siRNA targeted to *MDR1* gene is able to suppress P-gp expression and function, in an exquisitely selective way. Alternately, pharmacological modulation approaches using typical efflux- and influx-transporters inhibitors (e.g. verapamil and valspodar, or prazosin and procainamide, for P-gp and hOCT1, respectively) are also being investigated, despite the fact that these pharmacological inhibitors are known not to be selective for a single transport system. Anyhow, the importance of these drug transporter inhibitors on the cellular disposition of the next-generation anticancer drugs such as nilotinib (AMN107), dasatinib (BMS354825) or bortezomide has not been studied in details yet and deserves further evaluation. Concerning nilotinib, a work has just been published, which has assessed the cellular uptake of this new compound in GIST cell lines [24].

In our laboratory, we are therefore planning to perform several basic interrelated *in vitro* studies in this field: a) to assess the functional consequence of P-gp and hOCT1 on intracellular concentrations of imatinib and newer anticancer drugs; b) to compare their relative importance; c) to examine the possibilities of modulating their cellular concentrations by pharmacological agents; and finally d) to determine *in vitro* the importance, in these cellular models, of a possible intracellular metabolism on drug cellular disposition.

In that perspective, the following paragraphs will briefly describe the standard operating procedures planned for these future experiments, as an example of possible developments and prospects for research in the field of drug intracellular disposition.

6.3.2 Summary of the protocol

The experiments will be carried out by our research group using the cell culture facility provided by the collaborating Laboratory of Clinical Chemistry of our University hospital, using previously published methods, notably by our group [11,14,25-27]. Cells (e.g. either *MDR1+/MDR1-* Schinkel cells, *MDR1+/MDR1-* K562 cells, hOCT1+/hOCT1- HeLa cells[6] or hOCT1+ CEM[7] cells) will be incubated in 6-well plates for 18 h at 37°C, in 2 ml growth medium containing 10% FCS in the presence of the drug at a clinically relevant range of concentrations. All concentrations will be tested in duplicates, for all drugs tested.

Following incubation, a 300-µl aliquot will be removed to determine the extracellular drug concentration. The excess supernatant medium will be discarded and cell cultures will be washed 3 times with ice-cold PBS before extraction with 1 ml methanol/water 60 : 40 on a planar shaker by gentle agitation for 3 h. The methanolic extracts will be collected in Eppendorf vials and centrifuged at RT. The supernatant will finally be evaporated to dryness under nitrogen and the residue reconstituted in 200 µl methanol/water 60 : 40 prior to analysis using a LC-MS/MS method, derived form the ones developed for imatinib (§ 2.2) or anti-HIV drugs [28]. The three relevant drugs that we would like to study first in our comparative experiments are imatinib, nilotinib and dasatinib. It is planned that other STIs (bortezomid, lonafarnib, sunitinib, etc) will be also subsequently studied.

In the first step, the drugs will be incubated alone at the clinically relevant concentrations in the various cell cultures. In a second step, we will perform the same experiments in the presence of P-gp inhibitors (e.g. verapamil and valspodar) added to the growth medium of *MDR1+/MDR1-* cells. Drugs known to inhibit hOCT1 transport (e.g. prazosin and procainamide) will be tested in the relevant cell systems.

Intracellular CYP450-mediated metabolism of nilotinib in our cell cultures will also be examined using the dependant-scan features of the LC-MS/MS instrument.

The concentrations determined in the various extracts collected (intra- and extracellular) will be normalized on total proteins (or DNA) measurements, used as surrogates of cell counts. The determination of protein will be carried out according to the bicinchonic acid assay already described in § 6.1.1.3. The quantity of drugs per mg of protein will then be compared between the various conditions of the experiments (i.e. regarding the transporter expression, the

[6] HeLa cell line is the first continuously cultured human malignant cell line, derived from the cervical carcinoma of Mrs. Henrietta Lacks (one of several pseudonyms).
[7] CEM cells are a lymphoblastic cell line originally derived from a child with acute lymphoblastic leukemia (ALL).

concentration tested, the drug tested and the transporter inhibitor used). A multi-way ANOVA analysis will be used in order to assess the significance of these comparisons.

6.3.3 Expected outcomes

The proposed *in vitro* studies will thus bring additional knowledge on the intracellular disposition of first and second generation of the new anticancer drugs, notably allowing a head-to-head comparison of their respective cellular efflux/uptake in stringent *in vitro* experiments. More specifically, these studies will allow to answer the following questions: a) to what extent these transporters influence their intracellular accumulation; b) can drug intracellular accumulation be influenced by transporters modulators; and c) are the anticancer drugs under study subjected to a significant intracellular metabolism in these cell systems?

More generally, the implementation of the above-mentioned cellular models in our laboratory will bring additional knowledge on the cellular –and possibly tissular– disposition of these new drugs. It may have important implications in the perspective of clinical trials assessing the potential benefit of a TDM program for the emerging class of targeted-anticancer drugs.

6.4 References

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Chapter 7 Conclusions and perspectives

A new era of targeted therapy of cancer is emerging. The drugs used up to now against cancer have a narrow therapeutic index and the responses produced are often only palliative as well as unpredictable. Targeted therapy, introduced in recent years, is directed against cancer-specific molecules and signaling pathways and seems thus to be characterized by more limited non-specific toxicities. Tyrosine kinases are an especially important target as they play an important role in the modulation of growth factor signaling [1]. Imatinib is a promising example of this approach for treating *BCR-ABL*-positive leukemias and gastrointestinal stromal tumors. It is also currently investigated for the treatment of various types of cancer for which several tyrosine kinase proteins have been found to be involved in the appearance or progression of the malignancy [2].

Considering the large interindividual differences in the expression and function of the systems involved in imatinib disposition (e.g. CYP3A4, P-gp, hOCT1 and AGP), exposure to this drug can be expected to vary widely among patients. Anyway, it becomes increasingly clear that a patient's response to drugs is influenced not only by the genetic heterogeneity of the drug target, but also by the patients genetic background and environmental factors that influence drug systemic and cellular disposition in the body.

7.1 Discussion and conclusions

Imatinib and the new-targeted anticancer drugs (e.g. erlotinib, bortezomib, sunitinib, dasatinib, etc), unlike most anticancer chemotherapy regimens, must apparently be taken indefinitely. Despite the "magic bullet" term initially associated to these drugs, this aspect represents in fact new challenges regarding their long-term tolerability, resistance emergence, as well as their pharmacoeconomic impact.

The threat of resistance in patients has prompted numerous investigators to study all mechanisms whereby malignant cells develop resistance to imatinib. Some commonly known mechanisms of clinical resistance, such as the emergence of point mutation or genetic amplification of the target, have already been largely studied in the past years. Our own study has brought additional informations on various other potential resistance mechanisms, which may involve overexpression of various cellular drug transporters, metabolizing enzymes and plasma carrier proteins. As these systems show a high degree of polymorphisms and are subject to

numerous environment influences, they may be responsible for the demonstrated large interindividual variability in imatinib plasma and cellular pharmacokinetics. Such a variability in turn affects the maintenance of adequate drug levels, with direct implication on the treatment effectiveness and patient outcome. After the set-up and application of various analytical methods, the present study has been able to demonstrate some impacts of the above-mentioned systems (*in vitro* as well as *in vivo*, based on the clinical trial carried out). It has notably provided an original population pharmacokinetic model of imatinib integrating the impact of the carrier protein AGP.

Concerning the pharmacoeconomic impact of these new drugs, it is important to note that imatinib is indeed a rather expensive drug when compared to former anticancer drugs [3]. Hopefully for the CML patients, over 5 years, a patient in accelerated phase will, on average, accrue an additional 2.09 QALY (quality-adjusted life-years) with imatinib compared with conventional therapies, while patients in blast crisis will accrue an additional 0.58 QALY [4]. However, it has been shown that the cost estimates initially generated may be underestimated. The HTA program[1] in the United Kingdom has published a model showing more realistic values, with an incremental cost-effectiveness ratios for the chronic phase of $79'453–$525'330 per QALY gained [5]. For GIST patients, a recent study of the same program has, for instance, shown that the estimated median cost per QALY gained was $89'775-$172'333 after 2 years [6]. This represents consequent amounts and the pharmacoeconomic challenges of paying for all of the new anticancer agents will thus certainly become an area of increased attention [7]. Some authors are starting to recommend to authorities to be more willing to reimburse new cancer drugs for patients to benefit and to the pharmaceutical companies to be more prudent in their price setting [8]. If not, the availability of these drugs for patients will become more and more dependent on economic considerations and the gap between the 'haves' and 'have-nots' of the medical world will widen.

Despite of the various results obtained during this thesis work, and those largely presented in the literature to date, a number of issues such as imatinib disposition in various tissular or cellular compartments of the body, as well as its optimal clinical use, remain to be investigated. Gaining this knowledge could be useful not only for imatinib itself, but may certainly fruitfully apply to all the generation of new anticancer drugs.

In particular, the potential benefit of a therapeutic drug monitoring (TDM) program for imatinib and new related agents remains to be investigated. The important pharmacokinetic

[1] The Health technology assessment (HTA) program has for role to ensure that high-quality research information on the costs, effectiveness and broader impact of health technologies is produced in the most efficient way for those who use, manage and provide care in the National Health System of the UK.

variability demonstrated and quantified in Chapter 3, associated with the potential PK-PD relationship of imatinib (evaluated in Chapter 4) represent two arguments to evaluate further the potential benefit of a TDM program for this drug by well-controlled clinical trials.

7.2 Perspectives

7.2.1 Short-term perspectives

A number of additional pharmacogenetic polymorphisms with potential clinical consequences might be studied among the patient population of our study. It could be worth investigating of the polymorphism of the transport proteins hOCT1 and BCRP, for which imatinib has recently been demonstrated to be a substrate, and to determine to which extent these polymorphisms can explain some of the wide interindividual pharmacokinetic variability of imatinib (in part unexplained at present). Furthermore, performing the *in vitro* experiments on drug transporters described at the end of Chapter 6, is anticipated to bring additional knowledge on the cellular – and possibly tissular– disposition of these new drugs. It may have important implications in the perspective of clinical trials design, for assessing the potential benefit of a TDM program for the emerging class of targeted anticancer drugs.

Moreover, increasing exposure to the drug by inhibiting its efflux or its metabolism might potentially enhance its efficacy. This could particularly be the case for patients showing progressive resistance towards the drug at usual dosages. Such inhibitors would be drugs like ketoconazole (for CYP3A4) or verapamil (for P-gp). Their coadministration with imatinib (under the careful monitoring of plasma and intracellular concentrations) could potentially diminish both the interindividual variability and the intraindividual fluctuations in imatinib pharmacokinetics. Such an interaction would not only have a dose-sparing effect –with potential economic consequences–, but also increase the probability that the patient is exposed to adequate plasma concentrations, and that this translates into sufficiently high intracellular concentrations at the target site. Studies evaluating these issues are presently considered in our Division.

7.2.2 Mid-term perspectives

A direct clinical consequence of our project would be the development and validation of the therapeutic drug monitoring for imatinib, which could also be made available to all clinicians throughout Switzerland. However, whether individualization of imatinib dosage regimen based on routine drug level monitoring should be recommended for patients' management still deserves further confirmation in well-controlled trials. Such a validation of a TDM for imatinib (or other

new anticancer drug) is presently considered in our hospital under the name "Translational program for the evaluation of the role of Therapeutic drug monitoring of new anticancer drugs".

Combining indeed a TDM approach with an adequate pharmacogenetic approach (i.e. genotyping or phenotyping of the drug target in patients) would possibly provide a comprehensive set of parameters for the clinical management of gene-targeted therapy. This could be of direct help for clinicians at patients' bed site, in order to use such drug in a fully optimized and rational manner. It may help to individualize the dosing regimen before overt disease progression or observation of treatment toxicity, thus improving both the long-term therapeutic effectiveness and the tolerability of this drug (obtained moreover at lower global drug expenditures). Additionally, attention devoted to TDM could contribute to optimize patients' compliance (an important issue for a chronic inhibition of the target proteins) and somehow help to control the important costs associated with these new treatments. The large amount of data on the markers generated by all these techniques (especially pharmacogenetics) will need to be very well integrated and carefully interpreted. To that endeavor, bioinformatics will certainly be of great help if wisely used by trained specialists. It is noteworthy that some authors already state that we are entering the post-genome era, where use of bioinformatics will be crucial for managing all the data regarding oncology research and oncologic patient's treatments [9].

To conclude, we can say that the TDM of anticancer drugs is still in an early development phase, but the future years will probably be determinant for the evolution of this new concept and for its possible clinical applications. As "every patient is different", an approach that integrates clinical pharmacokinetics (i.e. TDM) and patient/tumor pharmacogenetics should allow to administer the right drug at the correct dose to the right patient.

7.3 References

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Appendices

The appendices relative to each chapter are ordered sequentially in the next pages.

Appendix 1.1

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Lausanne, le 24.6. 2002

ETUDE DE LA VARIABILITÉ DES CONCENTRATIONS PLASMATIQUES D'IMATINIB (GLIVEC®)

ET DE SA RELATION AVEC L'ACTIVITE DU CYTOCHROME P450 3A4

CHEZ DES PATIENTS ONCOLOGIQUES

Date d'envoi du protocole amendé : 24 juin 2002

Date prévue pour le début de l'étude : Début août 2002

Lieu de l'étude : Centre Pluridisciplinaire d'Oncologie, Division Spécialisée d'Hématologie, Laboratoire de la Division de Pharmacologie clinique, CHUV.

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INTRODUCTION

Mode d'action de l'imatinib

Imatinib et leucémies myéloïdes chroniques (LMC)

Le nouveau médicament antileucémique Glivec® (imatinib, STI571) a été annoncé aussi bien dans la presse professionnelle que grand-public comme le prototype d'une nouvelle génération d'agents anticancéreux [1-3, 6-8,13]. A l'opposé de la plupart des médicaments

anticancéreux conventionnels qui ont été découverts à travers un *screening* de l'activité cytotoxique de milliers de composés chimiques, l'imatinib a été conçu pour interagir spécifiquement avec une enzyme, une tyrosine kinase codée par l'oncogène *bcr-abl* qui caractérise les leucémies myéloïdes chroniques (LMC) arborant le chromosome Philadelphie.

Le chromosome Philadelphie, présent chez 95% des patients atteint de LMC, est formé à la suite d'un raccourcissement du chromosome 22 par une translocation réciproque entre le chromosome 9 et 22, ce qui conduit au remplacement du premier exon du gène *c-abl* par des séquences du gène *bcr*, résultant en l'expression de la protéine de fusion BCR-ABL (également appelée p210). Cette protéine possède une activité tyrosine kinase marquée, toujours sous forme activée, avec pour conséquence la phosphorylation de protéines effectrices conduisant à une prolifération cellulaire. Cette enzyme mutante induit ainsi la cellule à devenir cancéreuse. De nombreuses évidences expérimentales indiquent que la protéine BCR-ABL est un facteur majeur de la pathophysiologie de la LMC.

L'imatinib est particulièrement actif chez les patients souffrant de la forme précoce, chronique, de la LMC, comme l'a montré l'étude initiale avec 53 patients sur 54 montrant une réponse complète durable [1]. Les patients souffrant de la LMC au stade de la crise blastique répondent bien initialement, mais récidivent malgré la poursuite du traitement. Il n'y a pas encore à notre connaissance d'explications permettant de comprendre pourquoi des cellules résistantes émergent de la crise blastique, mais pas lors de la phase initiale chronique. Les cellules lors de ces deux phases présentent une morphologie et des sensibilités différentes. La phase chronique est constituée de neutrophiles qui ne se divisent plus et sont complètement spécialisés. A l'opposé, la multiplication et la dissémination de cellules précurseurs myéloïdes et lymphoïdes non spécialisées caractérise la phase blastique. Ces populations cellulaires semblent pourtant présenter *in vitro* en culture la même sensibilité à l'imatinib, mais il est concevable qu'*in vivo* des mutations ont lieu plus fréquemment dans des cellules qui se divisent plus souvent. D'autres mécanismes responsables de cette susceptibilité différente *in vivo* sont envisageables (voir infra).

Les études initiales réalisées sur une cohorte de patients indiquent que les réponses cliniques étaient corrélées à la possibilité d'atteindre des concentrations plasmatiques d'imatinib correspondant à celles connues pour inhiber l'activité enzymatique de BCR-ABL, suggérant que la sélection de la dose de médicament basée sur la pharmacocinétique du médicament apparaît comme une approche attractive.

Imatinib et tumeurs stromales d'origine digestive (GIST)

Les GIST sont des tumeurs mésenchymateuses du tractus gastro-intestinal, qui sont localisées à près de 70% dans l'estomac, et qui se trouvent avant tout chez des sujets d'âge moyen ou d'âge plus avancé. Les GIST constituent une forme rare de cancer, dont l'incidence est mal connue (25-50 cas par année en Suisse). On ne disposait d'aucune méthode thérapeutique efficace jusqu'à l'avènement de l'imatinib [4,5], qui a pu montrer un recul, parfois spectaculaire, de la tumeur chez près de deux tiers des 200 patients traités par Glivec aux USA et en Europe.

Il a pu être démontré que l'imatinib interagissait également avec une tyrosine kinase dénommée c-KIT, semblable à BCR-ABL, qui est à l'origine de la croissance des tumeurs stromales GIST. La protéine c-KIT se trouve dans certains tissus normaux, où elle joue une rôle dans la formation des cellules sanguines et la fonction intestinale. Les tumeurs GIST sont associées à une mutation de cette protéine qui conduit à une accélération de la croissance des cellules concernées. Les mécanismes de contrôle de la croissance normale perdent leur influence. Il semble ainsi que l'activation de la protéine c-KIT mutante est l'événement pathogénique décisif dans le développement des GIST.

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Les études réalisées jusqu'à ce jour semblent montrer que la réponse au traitement par le Glivec® est au moins aussi rapide lors de GIST que dans la LMC. Le Glivec® a conduit chez environ 80% des patients à une nette amélioration du tableau clinique. A l'heure actuelle, il faut cependant se garder de trop grands espoirs sur ce traitement : chez certains patients atteints de GIST, le Glivec® n'a donné aucun résultat, et on ne sait pas si les cellules cancéreuses continueront à répondre après une période de traitement prolongée, voire à vie.

Résistance à l'imatinib

Comme discuté, l'imatinib partage malheureusement une des caractéristiques des agents anticancéreux conventionnels : bien que la plupart des patients LMC répondent initialement au Glivec®, leurs cellules tumorales deviennent résistantes au cours du traitement et leur maladie récidive [10].

Les mécanismes de résistance ont été étudiés et identifiés dans le cas de la LMC : il s'agit soit d'une mutation ponctuelle de l'enzyme cible BCR-ABL qui peut ainsi fonctionner même en présence de l'imatinib, ou bien alors une multiplication du gène *brc-abl* codant pour celle-ci [15]. Ces copies surnuméraires du gène produisent une plus grande quantité d'enzyme BCR-ABL à laquelle le médicament ne peut pas faire face. Dans le cas où cette résistance est due à une amplification du gène *bcr-abl*, l'excès de l'enzyme BCR-ABL pourrait être donc en théorie contrecarrée par des doses plus élevées d'imatinib, dans le cas où il existe une corrélation claire entre la dose et la concentration du médicament au site d'action.

Dans d'autre cas, une seule mutation ponctuelle, toujours la même, change un seul acide aminé dans le site actif de l'enzyme et empêche l'imatinib de se fixer sur l'enzyme. A ce stade, il faut se poser la question si des concentrations suboptimales de façon chronique pourrait, par un processus de pression sélective, être responsable de l'apparition de tel mécanisme de résistance.

En effet, il a pu être démontré que des lignées cellulaires de LMC peuvent développer *in vitro* une résistance à l'imatinib après plusieurs mois de culture en présence de concentrations sous-thérapeutique d'imatinib, en montrant notamment une amplification du gène *bcr-abl*. [14,15].

Il est intéressant de relever que bien que les cellules générées lors de la crise blastique sont supposées être génétiquement instables, la réactivation ou l'amplification de BCR-ABL est *seule* responsable de la récidive [15]. Cette récidive n'est en effet pas due à l'apparition d'autres signaux oncogéniques secondaires, indiquant que cette tyrosine kinase reste tout au long du traitement une cible attractive pour combattre ce cancer.

A l'opposé du 5 fluoro-uracile (5-FU) et du méthotrexate (MTX) qui bloquent des enzymes indirectement impliquées dans la croissance de la cellule cancéreuse, l'imatinib agit spécifiquement sur la protéine responsable de la transformation maligne. Malgré cette différence fondamentale, la résistance à l'imatinib présente cependant des analogies avec la résistance au 5-FU et au MTX, à savoir une mutation ponctuelle de l'enzyme cible, ou une amplification du gène codant pour celle-ci. Mais il a été également démontré que la résistance au 5-FU peut aussi avoir lieu indirectement par la modulation ou l'altération des enzymes responsables de son *métabolisme* [14]. En principe, la résistance au STI-571 pourrait aussi avoir lieu indirectement.

En fait, la progression de la maladie chez les patients qui répondent initialement à l'imatinib semble associée à l'échec dans le maintien d'une inhibition effective continue de la tyrosine kinase BCR-ABL [15]. De façon intéressante, une étude préclinique, réalisée chez la souris chez laquelle une lignée cellulaire LMC en phase blastique avait été greffée, a montré que l'α-1-glycoprotéine acide (AAG), synthétisée en excès par le foie lors d'un processus inflammatoire aigu, peut se lier à l'imatinib et réduire ainsi son activité vis-à vis de BCR-ABL [21]. Il a été suggéré que cette liaison pourrait être modulée par une co-administration de

clindamycine qui entre en compétition pour la fixation à l'AAG [9]. Cette observation suggère que la résistance à l'imatinib peut être influencée par des facteurs modulant sa pharmacocinétique, et donc que cette résistance pourrait se développer indépendamment de la sensibilité de la tumeur.

Métabolisme de l'imatinib

L'imatinib est bien absorbé après administration orale et est fortement lié aux protéines plasmatiques. L'imatinib est métabolisé par le foie essentiellement par le cytochrome P450 3A4, et dans une moindre mesure par le CYP1A2, 2D6, 2C9 et 2C19 [16].

L'imatinib est un inhibiteur compétitif du CYP3A4 mais alternativement, l'administration simultanée d'inhibiteurs du CYP3A4 comme l'itraconazole ou l'érythomycine augmente les concentrations plasmatiques d'imatinib, alors que des inducteurs du CYP3A4 diminuent les concentrations du médicament.

Aucune donnée n'est disponible quant à l'utilisation d'imatinib chez des patients souffrant d'insuffisance hépatique ou rénale.

En considérant ses propriétés physicochimiques (basicité, lipophilie, etc.) et son comportement dans les liquides biologiques, il est concevable que l'imatinib soit également un substrat de la P-glycoprotéine, un transporteur membranaire qui expulse les médicaments à l'extérieur de la cellule. Cette protéine est diversement exprimée au niveau tissulaire (entérocytes, barrière hémato-encéphalique, etc.) et cellulaire. La P-glycoprotéine semble être particulièrement exprimée dans des populations de cellules souches, ce qui explique leur relative résistance aux agents toxiques et aux anticancéreux conventionnels notamment.

Il faut mentionner qu'aussi bien les enzymes hépatiques comme le CYP3A4, que les transporteurs membranaires (P-glycoprotéine) ont un niveau d'expression extrêmement variable d'un individu à l'autre, en partie dû à un polymorphisme génétique pour la P-glycoprotéine affectant sa régulation ou son fonctionnement [19,20].

Activité du Cytochrome P450 3A4

Le cytochrome P450 3A4 (CYP3A4) correspond à environ 30% du contenu total en cytochromes hépatiques et il contribue au métabolisme oxydatif de nombreux médicaments dont l'imatinib. Le CYP3A4 est également largement exprimé au niveau du tractus gastro-intestinal. L'activité du CYP3A4 peut varier d'au moins 10 fois entre individus, mais des études à large échelle sur cette variabilité inter- et intra-individuelle sont relativement rares. Cette différence d'activité du CYP3A4 est un des facteurs responsables de la grande variabilité pharmacocinétique intra- et inter-individuelle observée lors de traitement médicamenteux.

La détermination de l'activité enzymatique du CYP3A4 (phénotypage) peut être cliniquement utile. Le CYP3A4 présente non seulement un grande variabilité interindividuelle, mais il peut en outre être inhibé ou induit par des médicaments. Cela peut entraîner ainsi une élévation des concentrations plasmatiques des substrats de cette enzyme, tel que le Glivec®, et à des effets indésirables, ou alternativement à une réduction de ses concentrations sanguines et de ses effets thérapeutiques.

L'activité du CYP3A4 peut être mesurée par différents tests (*erythromycin breath test*, clairance plasmatique du midazolam, rapport 6-β-hydroxycortisol/cortisol urinaire). Parmi ceux-ci, la mesure du 6-β-hydroxycortisol/cortisol endogène dans l'urine est l'approche la plus attractive, car elle ne requiert pas l'administration de substances exogènes. Ce test non invasif ne nécessite que la récolte d'un spot urinaire.

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OBJECTIFS

Objectif principal

- Etudier la variabilité des concentrations plasmatiques d'imatinib chez des patients traités pour LMC ou GIST
- Etudier la variabilité des taux d'AAG chez des patients traités pour LMC ou GIST
- Déterminer l'activité enzymatique du CYP3A4 par la mesure du rapport cortisol/6βOH-cortisol urinaire, chez des patients traités pour LMC ou GIST

Objectifs secondaires

- Etudier la relation entre les concentrations plasmatiques d'imatinib, éventuellement modulées par les taux d'AAG et l'évolution clinique (récidives, échec initial/secondaire, effets indésirables). En d'autres termes, les récidives sont elles observées plus fréquemment chez des patients présentant des concentrations plasmatiques d'imatinib faibles. A contrario, les effets indésirables [11,12], le plus souvent modérés - mais qui peuvent nécessiter l'arrêt du traitement chez 5% des patients - sont-il observés chez des patients présentant des concentrations élevées d'imatinib.

DESSIN DE L'ETUDE

Etude ouverte, prospective, descriptive

SELECTION DES SUJETS

Il est prévu d'inclure dans cette étude environ 60 patients (hommes et femmes, sans limite d'âge), recevant du Glivec® pour une LMC ou un GIST et acceptant d'y participer.

Les sujets seront recrutés parmi les patients pris en charge par le Centre Pluridisciplinaire d'Oncologie et la Division spécialisée d'Hématologie.

CRITERE DE NON-INCLUSION

La prise d'autres médicaments que le Glivec® n'est pas un critère de non-inclusion (mais ils devront être clairement rapportés). Les substances inductrices ou inhibitrices du CYP3A4 ou de la P-Glycoprotéine seront activement recherchées dans l'anamnèse.

DEROULEMENT DE L'ETUDE

Lors d'une visite médicale faisant partie de la prise en charge habituelle, le but de l'étude sera exposé aux patients. Les patients intéressés recevront une information orale ainsi qu'un formulaire d'information précisant les objectifs et les détails de l'étude (annexe 1). Après signature du consentement informé, un échantillon sanguin (4 ml Monovettes® EDTA) sera prélevé en même temps que ceux prévus pour les dosages de routine (chimie, hématologie). L'obtention d'un profil sanguin chez une partie des patients serait souhaitable. Les investigateurs se réservent la possibilité de demander à quelques patients de fournir plusieurs prélèvements lors d'une même visite à leur convenance. Pour des raisons pratiques, un seul taux sera visé, en privilégiant dans la mesure du possible la prise lors de la phase d'élimination (prise de sang à 4 h au moins de la dernière dose (C_{max} de l'imatinib 1-4h après la dose). Il est prévu pour chaque patient la participation à au moins 2 prélèvements à ~3 mois d'intervalle (évaluation de la variabilité intra- et inter-individuelle).

L'**heure exacte** de la prise de sang, la **posologie quotidienne**, ainsi que la **dose**, la **date** et l'**heure exacte de la dernière** prise de Glivec® seront scrupuleusement reportés sur le formulaire « Demande de dosage de médicament du LCC » (ou sur un formulaire ad hoc

conçu pour l'étude) en mentionnant sous *Autres* « Glivec/STI571». Les autres médicaments pris par le patient seront également reportés.

Par ailleurs, il sera demandé aux patients un aliquot (« spot » d' environ 20-30 ml) d'urine dans un récipient ad-hoc. Comme seul le rapport des métabolites 6-β-hydroxycortisol/cortisol est considéré, le volume de la miction ne sera pas mesuré.

Ces échantillons (sang 4 ml et spot urinaire) seront obtenus chez les patients lors de chaque visite médicale de contrôle, mais au moins une fois tous les trois mois.

La Monovette® et l'échantillon urinaire seront acheminés avec le formulaire correspondant au desk de chimie clinique BH18 qui l'adressera au Laboratoire de Pharmacologie clinique (LPCL).

Stockage des échantillons

Les échantillons sanguins seront immédiatement centrifugés (1000 g, +4°C, 10 min) au LPCL et le plasma ainsi que l'échantillon urinaire seront stockés à -25°C avant analyse par HPLC.

La mesure de l'AAG sera effectuée sur le plasma au LCC.

Le culot (érythrocytes et *buffy coat*) obtenus après centrifugation des échantillons sanguins sera conservé en 3 aliquots en vue d'une analyse ultérieure par PCR pour le polymorphisme de la PgP [19,20], d'un autre transporteur, des cytochromes P450, ou pour étudier l'augmentation de l'expression de la BRC-ABL, ou de sa mutation.

Les prélèvements d'ADN à partir des échantillons sanguins seront codés (deuxième lettre du nom, première lettre du prénom, date) pour garantir l'anonymat des patients. Les résidus des échantillons et/ou les échantillons non analysés seront détruits une année après la fin de l'étude mais au plus tard en janvier 2007.

Analyses des échantillons

Le 6-β-hydroxycortisol/cortisol dans les échantillons urinaires seront dosés par HPLC (extraction liquide/solide, en utilisant deux méthodes de détection (UV et LC-MS/MS; laboratoire PCL). L'analyse de l'imatinib dans le plasma sera analysé par HPLC (laboratoire PCL). Le dosage de l'AAG est disponible au LCC.

Acquisition et récolte des données cliniques.

Du point de vue des *outcomes*, l'hypothèse la plus raisonnable est que la concentration d'imatinib permet de prédire l'évolution clinique *dans le mois qui suit*. Il est donc prévu de relever comme *outcome* clinique l'évolution à partir de la prise de sang et durant le mois suivant. Seront considérés les marqueurs de la progression tumorales (numérotation leucocytaire pour la LMC, grandeur de la tumeur digestive pour le GIST, etc.). La réponse sera codée sur l'échelle : régression-stabilité-progression.

En ce qui concerne les effets indésirables, il est habituel d'utiliser les échelles OMS de toxicité (grades 0-4 pour une série de symptômes et de marqueurs biologiques standard). Il serait idéal que le praticien puisse les mesurer eux aussi au moment de la prise et 1 mois plus tard.

Version 24-6-2002

Analyse des données et interprétation des résultats.

Critères d'évaluation

1. Indice d'activité enzymatique du CYP3A4 (rapport 6-β-hydroxycortisol/cortisol urinaire)
2. Concentrations d'imatinib en fonction de la dose et du temps
3. taux d'AAG
4. apparition de résistance au traitement ou de récidive (selon l'échelle : régression-stabilité-progression)
5. apparition d'effets indésirables liés au traitement d'imatinib
6. expression de la BRR-ABL et/ou de sa mutation
7. relations entre points 1 et 2, points 2 et 3 et points 2-3 et 4, 2-3 et 5, 2-3 et 6

Analyse cinétique et statistique

Pharmacocinétique de population, par une estimation Bayésienne de l'AUC comme mesure de l'exposition, puisque le nombre de points pour chaque patient est en général limité.

CONSENTEMENT

Un consentement écrit sera demandé à chaque sujet après information orale et lecture de la feuille d'information ci-jointe.

PROCEDURE

Cette investigation se déroule à l'occasion des visites de contrôle de routine et nécessite le don par chaque patient d'un échantillon de sang (4 ml) et d'un échantillon d'urine.

Le temps effectif que les patients devront consacrer à cette étude est d'environ 10 min (lecture de la feuille d'information, signature de la feuille de consentement, prise de sang et récolte d'un échantillon urinaire).

SURVEILLANCE

Les patients seront suivi dans le cadre de la prise en charge habituelle de leur pathologie, il n'est prévu aucune surveillance particulière supplémentaire pour cette étude.

EVALUATION DES RISQUES

- Cette étude exigera la prise d'un tube de sang supplémentaire par visite. Le volume total de sang total prélevé durant toute l'étude (durée prévue : 1½ ans) sera d'environ 30 ml (1 prélèvement de 4 ml tous les 3 mois).
- La récolte d'un spot urinaire ne représente qu'un désagrément supplémentaire modeste.

DEDOMMAGEMENT

Il n'est pas prévu de dédommagement pour cette étude.

Version 24-6-2002

FINANCEMENT

Cette étude ne bénéficie d'aucun soutien de l'industrie pharmaceutique. Le coût des tubes de récolte (Monovettes, récipients pour récolte urinaire, etc.) seront pris en charge par la Division d'Hématologie et par le CePO. Il est estimé que le coût total du développement de la méthode analytique par HPLC et des analyses des échantillons par le Laboratoire de Pharmacologie clinique, ainsi que le dosage de l'AAG par le Laboratoire de Chimie clinique devrait être inférieur à CHF 10'000.- (environ 250 échantillons à 30.- pour l'imatinib et l'AAG) et seront pris en charge dans le cadre du budget d'exploitation du Laboratoire de Pharmacologie clinique.

CALENDRIER

- Soumission à la commission d'éthique: mai 2002.
- Début de l'étude : juin 2002.
- Fin de l'étude : automne 2003.

Version 24-6-2002

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Appendix 1.2

Version 24-6-2002

ANNEXE 1 Lausanne, le 24 juin 2002

ETUDE DE LA VARIABILITÉ DES CONCENTRATIONS PLASMATIQUES D'IMATINIB (GLIVEC®) ET DE SA RELATION AVEC L'ACTIVITE DU CYTOCHROME P450 3A4 CHEZ DES PATIENTS ONCOLOGIQUES

FEUILLE D'INFORMATION

BUT de L'ETUDE

Il a été observé que les médicaments comme le Glivec®, qui sont métabolisés par certaines voies enzymatiques, présentent, pour une posologie identique, des taux sanguins très variables d'une personne à l'autre.

Il se pourrait que des concentrations trop faibles ou trop fortes de Glivec®, liées à cette grande variabilité inter-individuelle, soit à l'origine de l'apparition d'une résistance au traitement ou du développement d'effets indésirables. C'est cette hypothèse que nous désirons tester par cette étude.

Vous ne retirerez sans doute pas de bénéfice direct de votre participation à cette étude, dont les résultats pourraient cependant améliorer la prise en charge ultérieure des patients souffrant de la même affection que vous.

Nous nous proposons donc d'analyser les concentrations sanguines du médicament Glivec®. Nous nous intéressons en particulier à étudier si les concentrations varient beaucoup d'un patient à l'autre et/ou au cours du traitement et si ces concentrations peuvent expliquer les résultats obtenus en terme d'efficacité ou de tolérance.

A cet effet, nous profitons de votre présence au CHUV pour vous demander si vous êtes disposé(e) à participer à cette étude. Votre participation consiste à fournir un échantillon de sang supplémentaire (environ 4 ml), prélevé à l'occasion de vos visites médicales, et destiné à la mesure des concentrations sanguines de Glivec®. Il nous serait utile que quelques patients acceptent de fournir plusieurs échantillons.

Nous souhaitons également évaluer l'activité d'un système enzymatique, appelé CYP450 3A4, impliqué entre autres dans le métabolisme du Glivec®. A cet effet nous souhaiterions obtenir de votre part un échantillon d'urine à l'occasion de vos visites de contrôle. Cette urine permettra de mesurer le rapport cortisol/6-β-hydroxy-cortisol, qui est un reflet de cette activité enzymatique.

Le sang prélevé dans le cadre de cette étude pourra également être utilisé pour l'analyse des gènes codant pour des protéines régulant vraisemblablement le passage du Glivec® dans les cellules, et son élimination, ainsi que pour la protéine qui est la cible pharmacologique du Glivec®.

Les échantillons d'ADN prélevés à partir du sang seront codés pour garantir l'anonymat. Ces échantillons seront détruits une année après la fin de l'étude, mais au plus tard en janvier 2007.

Vous êtes entièrement libre de participer ou non à cette étude. Votre décision n'influencera en rien votre prise en charge médicale. Si vous décidez d'y participer, vous pouvez à tout moment interrompre votre participation sans avoir à vous justifier.

Cette étude exige la prise d'un tube de sang supplémentaire par visite (4 millilitres). Le volume total de sang total prélevé durant toute l'étude sera fonction de la durée de votre traitement. Si vous deviez participer à 1 prélèvement de sang supplémentaire chaque 3 mois et durant 1 an et demi, le volume de sang prélevé équivaudra à environ 30 ml (par comparaison, la quantité de sang prélevée lors d'un don de sang à la Croix-Rouge est de 400-450 ml).

La récolte d'un spot urinaire ne représente qu'un désagrément supplémentaire modeste.

Pr Serge Leyvraz Dr M. Duchosal Dr Anne Rosselet Dr Laurent Decosterd

Version 24-6-2002

ETUDE DE LA VARIABILITÉ DES CONCENTRATIONS PLASMATIQUES D'IMATINIB (GLIVEC®)
ET DE SA RELATION AVEC L'ACTIVITE DU CYTOCHROME P450 3A4
CHEZ DES PATIENTS ONCOLOGIQUES

FEUILLE DE CONSENTEMENT

Le soussigné

- confirme avoir été informé sur les objectifs et le déroulement de l'étude ci-dessus et avoir reçu une copie de la feuille d'information.
- affirme avoir lu attentivement et compris les informations écrites fournies en annexe, informations à propos desquelles il a pu poser toutes les questions qu'il souhaitait.
- atteste qu'un temps de réflexion suffisant lui a été accordé.
- a été informé du fait qu'il pouvait interrompre à tout instant sa participation à cette étude sans préjudice d'aucune sorte et sans avoir à fournir de raison.
- a été informé que la confidentialité des données recueillies pendant l'étude lui est garantie.

Le soussigné accepte de son plein gré et sur une base volontaire de prendre part à cette étude.

Lausanne, le .....

Nom et prénom du patient .....

Signature : .....

Nom et prénom du médecin .....

Signature : .....

Appendix 1.3

UNIVERSITE DE LAUSANNE

FACULTE DE MEDECINE
COMMISSION D'ETHIQUE
DE LA RECHERCHE CLINIQUE
RUE DU BUGNON 21-CH-1005 LAUSANNE

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Prof. J. Biollaz
Médecin Chef
Département de médecine
Division de pharmacologie clinique
CHUV
1011 Lausanne

Lausanne, le 28 juin 2002

Avis de la Commission d'Ethique de la recherche clinique

Monsieur et cher Collègue,

Lors de sa séance du **4 juin 2002**, la Commission d'Ethique de la recherche clinique, Sous-Commission II (dans la composition détaillée en page 4) a procédé à une évaluation approfondie du projet de recherche désigné ci-après :

Protocole 100/02 : Etude de la variabilité des concentrations plasmatiques d'imatinib (Glivec®) et de sa relation avec l'activité du cytochrome P450 3A4 chez des patients oncologiques

Investigateur :

Prof. J. Biollaz
Médecin Chef
Département de médecine
Division de pharmacologie clinique
CHUV
1011 Lausanne

La Commission d'Ethique de la recherche clinique, Sous-Commission II, base son appréciation sur les documents soumis les 10.05.2002 et 24.06.2002, soit :

- Protocole en français (version 24.06.2002) daté du 24 juin 2002
- Feuille d'information (version 24.06.2002) datée du 24 juin 2002
- Feuille de consentement (version 24.06.2002) daté du 24 juin 2002

☒ procédure ordinaire ☐ procédure simplifiée ☐ évaluation ultérieure

La Commission d'Ethique arrête l'**avis** suivant :

☒ A **Avis positif**
☐ B **Avis positif assorti de recommandations** (v. page 3 et suiv.)
Information écrite à la Commission d'éthique suffisante ☐
☐ C **Avis conditionnel** (v. page 3 et suiv.)
Evaluation ultérieure par la Commission d'éthique nécessaire ☐
Information écrite à la Commission d'éthique suffisante ☐
☐ D **Avis négatif motivé (et explication pour réexamen)** (v. page 3 et suiv.)
☐ E **Avis justifié de ne pas entrer en matière** (v. page 3 et suiv.)

L'avis s'applique également aux autres investigateurs mentionnés dans la demande d'évaluation, travaillant dans des sites de recherche relevant du champ de compétence de la Commission d'Ethique de la recherche clinique.

Pour mémoire : **Obligations de l'investigateur**

- Les produits testés et de comparaison (médicaments et dispositifs médicaux) doivent être fabriqués, évalués et utilisés conformément aux règles de l'art visant à en garantir la qualité et la sécurité.
- Devoir de signaler: a) immédiatement tout événement indésirable grave (serious adverse events)
  b) toute information devenant disponible au cours de l'essai et ayant des conséquences directes pour la sécurité des sujets et la poursuite de l'essai
  c) Modification du protocole
  d) Fin ou arrêt prématuré de l'essai
- Rapport intermédiaire: une fois par année
- Notification d'essais de médicaments auprès de l'OICM et de dispositifs médicaux auprès de l'OFSP (en cas d'étude sponsorisée, cette tâche incombe au promoteur)
- Rapport final

Emolument : Fr. 100.--

La Commission d'éthique, Sous-Commission II :

Dr Fulgencio Gómez, PD, MER
Président

Appendix 1.4

Version 24-7-2003

Lausanne, le 24 juillet 2003

ETUDE DE LA VARIABILITÉ DES CONCENTRATIONS PLASMATIQUES D'IMATINIB (GLIVEC®) ET DE SA RELATION AVEC L'ACTIVITE DU CYTOCHROME P450 3A4 CHEZ DES PATIENTS ONCOLOGIQUES

FEUILLE D'INFORMATION POUR LES PATIENTS SUIVANT UNE ETUDE CINETIQUE COMPLETE

BUT de L'ETUDE

Il a été observé que les médicaments comme le Glivec®, qui sont métabolisés par certaines voies enzymatiques, présentent, pour une posologie identique, des taux sanguins très variables d'une personne à l'autre.

Il se pourrait que des concentrations trop faibles ou trop fortes de Glivec®, liées à cette grande variabilité inter-individuelle, soit à l'origine de l'apparition d'une résistance au traitement ou du développement d'effets indésirables. C'est cette hypothèse que nous désirons tester par cette étude.

Vous ne retirerez sans doute pas de bénéfice direct de votre participation à cette étude, dont les résultats pourraient cependant améliorer la prise en charge ultérieure des patients souffrant de la même affection que vous.

Nous nous proposons donc d'analyser les concentrations sanguines du médicament Glivec®. Nous nous intéressons en particulier à étudier si les concentrations varient beaucoup d'un patient à l'autre et/ou au cours du traitement et si ces concentrations peuvent expliquer les résultats obtenus en terme d'efficacité ou de tolérance.

A cet effet, nous profitons de votre présence au CHUV pour vous demander si vous êtes disposé(e) à participer à une journée d'étude supplémentaire dans le cadre de l'étude que vous effectuez déjà. Votre participation consiste actuellement à fournir un échantillon de sang supplémentaire (environ 4 ml), prélevé à l'occasion de vos visites médicales, et destiné à la mesure des concentrations sanguines de Glivec®. Il nous serait utile que quelques personnes acceptent de nous fournir plusieurs échantillons sanguins durant une même journée, cela dans le but de mieux interpréter les résultats déjà obtenus au cours de notre étude. Si vous acceptez de prendre part à cette extension de l'étude, vous serez acceuilli durant une journée dans les locaux de la Division de Pharmacologie clinique. Le matin de l'étude, l'on vous placera un dispositif de prélèvement sanguin sur l'avant-bras. Deux premiers tubes de sang seront alors prélevés, puis vous pendrez votre médicament. Ensuite, des prélèvements de sang (environ 4 ml chacun) destinés au dosage du Glivec® dans le plasma seront effectués toutes les heures durant 8 heures. De plus des prélèvements de sang (environ 20 ml) destinés au dosage de ce même médicament à l'intérieur de vos globules blancs seront effectués après 2, 4 et 6 heures. À noter encore, que pour cette étude supplémentaire, un petit-déjeuner et un repas de midi standard vous seront offerts sur place. Durant cette journée, le montant total de sang prélevé sera d'environ 120 ml.

Le sang prélevé dans le cadre de cette étude pourra également être utilisé pour l'analyse des gènes codant pour des protéines régulant vraisemblablement le passage du Glivec® dans les cellules, et son élimination, ainsi que pour la protéine qui est la cible pharmacologique du Glivec®.

Les échantillons d'ADN prélevés à partir du sang seront codés pour garantir l'anonymat. Ces échantillons seront détruits une année après la fin de l'étude, mais au plus tard en janvier 2007.

Vous êtes entièrement libre de participer ou non à cette étude. Votre décision n'influencera en rien votre prise en charge médicale. Si vous décidez d'y participer, vous pouvez à tout moment interrompre votre participation sans avoir à vous justifier.

Pr Serge Leyvraz Dr M. Duchosal Dr Anne Rosselet Dr Laurent Decosterd

Version 24-7-2003

ETUDE DE LA VARIABILITÉ DES CONCENTRATIONS PLASMATIQUES D'IMATINIB (GLIVEC®)
ET DE SA RELATION AVEC L'ACTIVITE DU CYTOCHROME P450 3A4
CHEZ DES PATIENTS ONCOLOGIQUES

FEUILLE DE CONSENTEMENT POUR PATIENTS SUIVANT UNE ÉTUDE CINÉTIQUE COMPLÈTE

Le soussigné

- confirme avoir été informé sur les objectifs et le déroulement de l'étude ci-dessus et avoir reçu une copie de la feuille d'information.
- affirme avoir lu attentivement et compris les informations écrites fournies en annexe, informations à propos desquelles il a pu poser toutes les questions qu'il souhaitait.
- atteste qu'un temps de réflexion suffisant lui a été accordé.
- a été informé du fait qu'il pouvait interrompre à tout instant sa participation à cette étude sans préjudice d'aucune sorte et sans avoir à fournir de raison.
- a été informé que la confidentialité des données recueillies pendant l'étude lui est garantie.

Le soussigné accepte de son plein gré et sur une base volontaire de prendre part à cette étude.

Lausanne, le .....

Nom et prénom du patient .....

Signature : .....

Nom et prénom du médecin .....

Signature : .....

Appendix 1.5

UNIVERSITE DE LAUSANNE

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Prof. J. Biollaz
Médecin Chef
Département de médecine
Division de pharmacologie clinique
CHUV
1011 Lausanne

Lausanne, le 11 août 2003

Protocole 100/02 : Etude de la variabilité des concentrations plasmatiques d'imatinib (Glivec®) et de sa relation avec l'activité du cytochrome P450 3A4 chez des patients oncologiques

Monsieur et cher Confrère,

Au nom de la Commission d'Ethique, je vous remercie de m'avoir adressé votre lettre du 24 juillet 2003 concernant le protocole susmentionné ainsi que les documents suivants :

- **Feuille d'information pour patients suivant une étude cinétique complète, version 24-7-2003**
- **Feuille de consentement pour patients suivant une étude cinétique complète, version 24-7-2003**
- **Rapport intermédiaire et final du 28.06.2003**

Ces documents ne posent pas de problème éthique.

La Commission en a pris bonne note et les accepte.

En vous remerciant de m'avoir soumis ces documents, je vous prie d'agréer, Monsieur et cher Collègue, mes salutations les meilleures.

Dr Fulgencio Gomez, PD et MER
Président

Appendix 3.1

CENTRE HOSPITALIER UNIVERSITAIRE VAUDOIS
DEPARTEMENT DE MEDECINE

Centre coordonné d'oncologie ambulatoire (CCO)
Division et laboratoire central d'Hématologie (HEM)
Division de Pharmacologie clinique (PCL)
1011 **Lausanne**

Laboratoire PCL: BH 18.218, tél: 44 271, bip: 744 334

PATIENT
(ou étiquette DITO à code barre)

Nom, Prénom : ..... **Sexe** : ❑ F ❑ M

Date de naissance :

Service : ..... **Tél.** : .....

N° d'admission : .....

REQUÉRANT (médecin et service, téléphone)

Nom du médecin : .....

Service : ..... **Bip** : .....

DOSAGE DE L'IMATINIB (GLIVEC®)

Étude de la variabilité des concentrations plasmatiques d'imatinib et de sa relation avec l'activité du CYP3A4 chez des patients oncologiques

INFORMATIONS INDISPENSABLES

PRÉLÈVEMENT : **Date effective** : **Heure** : (h : min)

TRAITEMENT D'IMATINIB (GLIVEC®) : **Date dernière prise** : **Heure** : (h : min)

Date début traitement :

Dernier chgt posologie :

Dose : mg x/j (autre schéma à préciser sous commentaires)

POIDS DU PATIENT : kg

AUTRES MÉDICAMENTS : .....
(pris sur la dernière semaine, ...
y.c. en automédication) .....
.....

FONCTIONS ANORMALES : ❑ Atteinte cardiaque : ..... ❑ Atteinte hépatique : .....
(préciser) ❑ Atteinte rénale : .....

DIAGNOSTIC : ❑ Leucémie myéloïde chronique (LMC) ❑ Tumeur stromale gastro-intestinale (GIST) ❑ Autre : .....

RÉPONSE CLINIQUE : **(Date dernière évaluation** : **)**

LMC : **Réponse hématologique** : ❑ complète ❑ partielle ❑ absente **Phase** : ❑ chronique ❑ accélérée ❑ blastique
Réponse cytogénétique : % cellules Ph(+) **Réponse moléculaire** : % cellules BCR-ABL

GIST : **Réponse tumorale** (RECIST) : ❑ rémission complète ❑ rémission partielle ❑ stabilité ❑ progression ❑ rechute

EFFETS INDÉSIRABLES OBSERVÉS SUR LA DERNIÈRE SEMAINE (indiquer éventuellement la sévérité) :

❑ Nausées :	❑ Vomissements :	❑ Diarrhées : .....
❑ Douleurs abdominales :	❑ Oedèmes/Rétention hydr. :	❑ Dermatite/Rash cutané : .....
❑ Myalgies :	❑ Crampes musculaires :	❑ Arthralgies : .....
❑ Céphalées :	❑ État fébrile :	❑ Fatigue : .....
❑ Anorexie :	❑ Prise de poids :	❑ Anémie : .....
❑ Neutropénie :	❑ Thrombocytopénie :	❑ Autre : .....

COMMENTAIRES ET PRÉCISIONS (posologie particulière, demande spécifique, comorbidité, etc.) :

.....
.....

Bon à joindre au prélèvement sanguin (*5 ml EDTA-K*), accompagné du spot urinaire (*10 ml*), et à envoyer sans délai au *Desk du LCC* – BH 18, PP 439

Version 1.0 / 14.10.2002 / PCL

Appendix 3.2

CHUV **CENTRE HOSPITALIER UNIVERSITAIRE VAUDOIS**
DEPARTEMENT DE MEDECINE

Division de Pharmacologie clinique (PCL)
1011 **Lausanne**
Laboratoire PCL: BH 18.218, tél: 44 271, bip: 744 334

PATIENT N° : ☐

Nom, Prénom : ..... **Sexe** : ❑ F ❑ M
Date de naissance : ☐☐☐☐☐☐
Service : .....

DOSAGE DE L'IMATINIB (GLIVEC®)

Étude cinétique complète chez 6 patients oncologiques

TRAITEMENT D'IMATINIB (GLIVEC®) : **Date avant-dernière prise** : ☐☐☐☐☐☐ **Heure** : ☐☐☐☐ (h : min)

Date prise du jour : ☐☐☐☐☐☐ **Heure** : ☐☐☐☐ (h : min)

Heure de prise habituelle : ☐☐☐☐ (h : min)

Date début traitement : ☐☐☐☐☐☐

Dernier chgt posologie : ☐☐☐☐☐☐

Dose : ☐☐☐☐ mg ☐☐ x/j (autre schéma à préciser sous commentaires)

POIDS DU PATIENT : ☐☐☐ kg

PRÉLÈVEMENTS (h : min) :

Plasma :		**PBMC :**		**Urine :**	
P0	☐☐☐☐	M0	☐☐☐☐	U0	☐☐☐☐
P1	☐☐☐☐	M2	☐☐☐☐		
P2	☐☐☐☐	M4	☐☐☐☐		
P3	☐☐☐☐	M6	☐☐☐☐		
P4	☐☐☐☐				
P5	☐☐☐☐				
P6	☐☐☐☐				
P7	☐☐☐☐				
P8	☐☐☐☐				

REPAS : Déjeuner : ☐☐☐☐ (h : min) Dîner : ☐☐☐☐ (h : min)

AUTRES MÉDICAMENTS : ....
(pris sur la dernière semaine, ...
y.c. en automédication) ....

FONCTIONS ANORMALES : ❑ Atteinte cardiaque : ..... ❑ Atteinte hépatique : .....
(préciser) ❑ Atteinte rénale : .....

DIAGNOSTIC : ❑ Leucémie myéloïde chronique (LMC) ❑ Tumeur stromale gastro-intestinale (GIST) ❑ Autre : .....

RÉPONSE CLINIQUE : **(Date dernière évaluation** : ☐☐☐☐☐☐ **)**

LMC : **Réponse hématologique** : ❑ complète ❑ partielle ❑ absente **Phase** : ❑ chronique ❑ accélérée ❑ blastique
Réponse cytogénétique : ☐☐☐ % cellules Ph(+) **Réponse moléculaire** : ☐☐☐ % cellules BCR-ABL

GIST : **Réponse tumorale** (RECIST) : ❑ rémission complète ❑ rémission partielle ❑ stabilité ❑ progression ❑ rechute

EFFETS INDÉSIRABLES OBSERVÉS SUR LA DERNIÈRE SEMAINE (indiquer éventuellement la sévérité) :

❑ Nausées :	❑ Vomissements :	❑ Diarrhées : .....
❑ Douleurs abdominales :	❑ Oedèmes/Rétention hydr. :	❑ Dermatite/Rash cutané : .....
❑ Myalgies :	❑ Crampes musculaires :	❑ Arthralgies : .....
❑ Céphalées :	❑ État fébrile :	❑ Fatigue : .....
❑ Anorexie :	❑ Prise de poids :	❑ Anémie : .....
❑ Neutropénie :	❑ Thrombocytopénie :	❑ Autre : .....

COMMENTAIRES ET PRÉCISIONS (posologie particulière, demande spécifique, comorbidité, etc.) :

.....

Version 1.0 / 24.11.2004 / PCL

Appendix 3.3

Derivation of C_u (imatinib free concentration; equation (11)), assuming the saturable binding of imatinib onto AGP (considered as the main binding protein (Gambacorti-Passerini *et al*, 2000; reference [82] in Chapter 1):

$$\text{imatinib}_{\text{unbound}} + \text{AGP}_{\text{unbound}} \leftrightarrows \text{imatinib}_{\text{bound}} \cdot \text{AGP}_{\text{bound}} \quad (16)$$

Applying the law of mass action gives (u = unbound; b = bound):

$$K_d = \frac{C_u \cdot L \cdot AGP_u}{C_b} \quad (17)$$

while:

$$L \cdot AGP_{tot} = C_b + L \cdot AGP_u \quad (18)$$

Combining (17) and (18) thus provides an expression for C_u:

$$C_u = \frac{K_d \cdot C_b}{L \cdot AGP_{tot} - C_b} \quad (19)$$

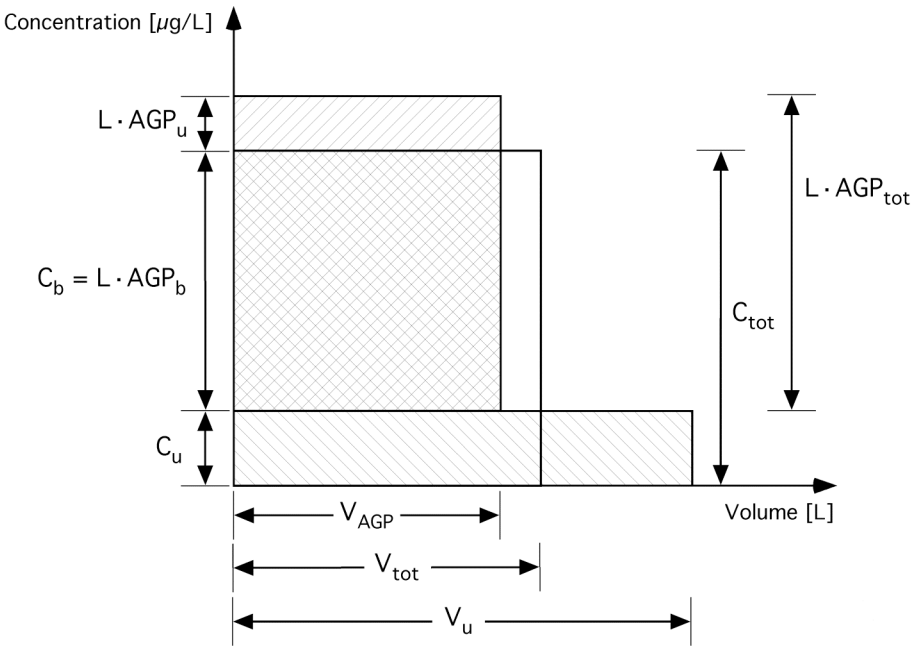
Therefore, considering that C_{tot} is equal to the sum of C_u and C_b (i.e. $C_b = C_{tot} - C_u$), we can write:

$$C_u^{\,2} - C_u \cdot (C_{tot} - K_d - L \cdot AGP_{tot}) - K_d \cdot C_{tot} = 0 \quad (20)$$

Finally, solving this equation according to C_u provides the expression of C_u given in equation (11).

Appendix 3.4

To express Vd as a function of AGP_{tot} and C_{tot}, it is useful to figure out the distribution of imatinib according to the following Figure (assuming that AGP is the main binding protein (Gambacorti-Passerini *et al*, 2000; reference [82] in Chapter 1):



Representation of imatinib distribution, regarding concentrations and volumes (V_{tot} = Vd = total imatinib volume of distribution, V_{AGP} = total AGP plasma volume, V_u = free imatinib volume, C_{tot} = total imatinib concentration, C_u = free imatinib concentration, C_b = concentration of imatinib bound to AGP, AGP_{tot} = total AGP concentration, AGP_u = free AGP concentration, AGP_b = concentration of AGP bound to imatinib, L = scaling factor)

Assuming this, it follows that the total unbound and AGP-bound volumes are given by:

$$V_{tot} = \frac{A_{tot}}{C_{tot}} \tag{21}$$

$$V_u = \frac{A_u}{C_u} \tag{22}$$

$$V_{AGP} = \frac{A_b}{C_b} \tag{23}$$

Assuming that the total quantity of imatinib (A_{tot}) is equal to the sum of the unbound (A_u) and bound (A_{bound}) quantities, we can write:

$$C_{tot} \cdot V_{tot} = C_u \cdot V_u + C_b \cdot V_{AGP} \tag{24}$$

Therefore, considering that C_{tot} is given by the sum of C_u and C_b (i.e. $C_u = C_{tot} - C_b$) it follows:

$$C_b = \frac{C_{tot} \cdot (V_{tot} - V_u)}{V_{AGP} - V_u} \tag{25}$$

Furthermore, assuming the law of mass action and rewriting equation (19) according to C_b gives:

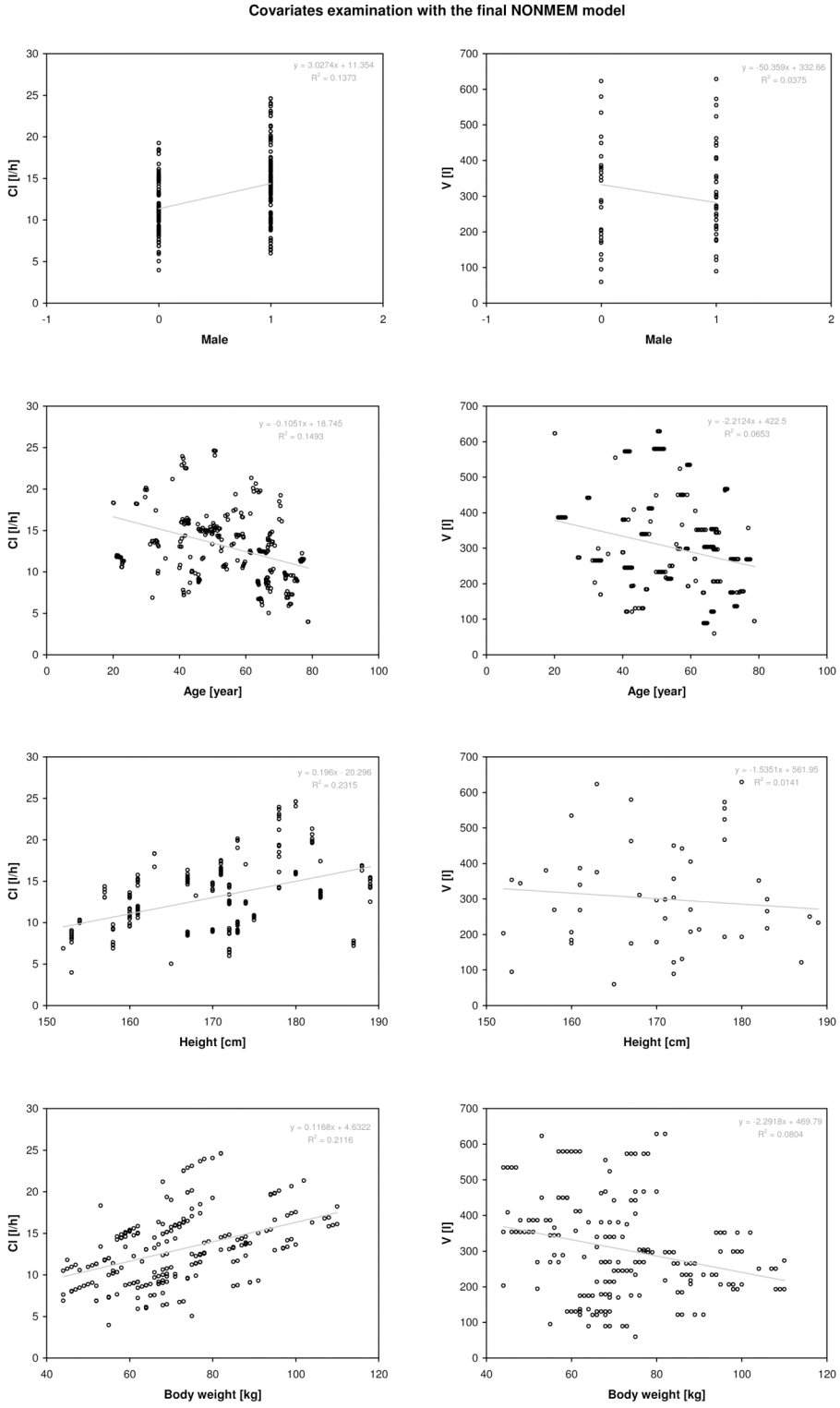
$$C_b = \frac{C_u \cdot L \cdot AGP_{tot}}{C_u + K_d} \tag{26}$$

Hence, considering again that C_u is given by $C_{tot} - C_b$ equation (26) can be expressed as:

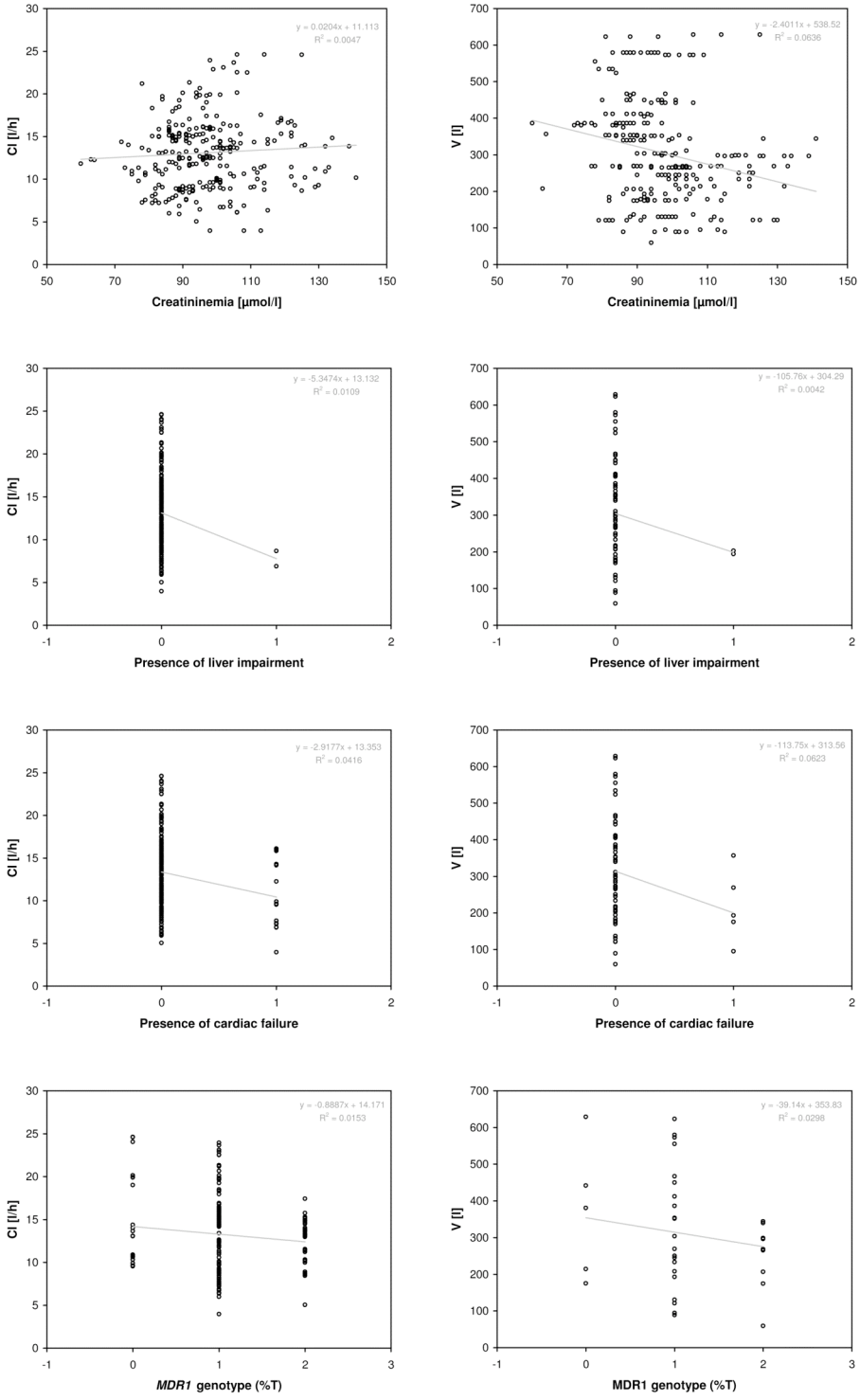
$$C_b^{\,2} - C_b \cdot (C_{tot} + K_d + L \cdot AGP_{tot}) + C_{tot} \cdot L \cdot AGP_{tot} = 0 \tag{27}$$

Substituting (25) into (27) and resolving according to V_{tot} gives equation (11), which expresses Vd (the ration between A_{tot} and CL_{tot}) as a function of AGP_{tot} and C_{tot}, with parameters V_{AGP}, V_u, K_d and L. The calculations were checked using Mathematica® (version 5.2, Wolfram Research Inc., Champaign, USA). Interestingly, equation (12) depicts an oblique hyperbole with one horizontal asymptote.

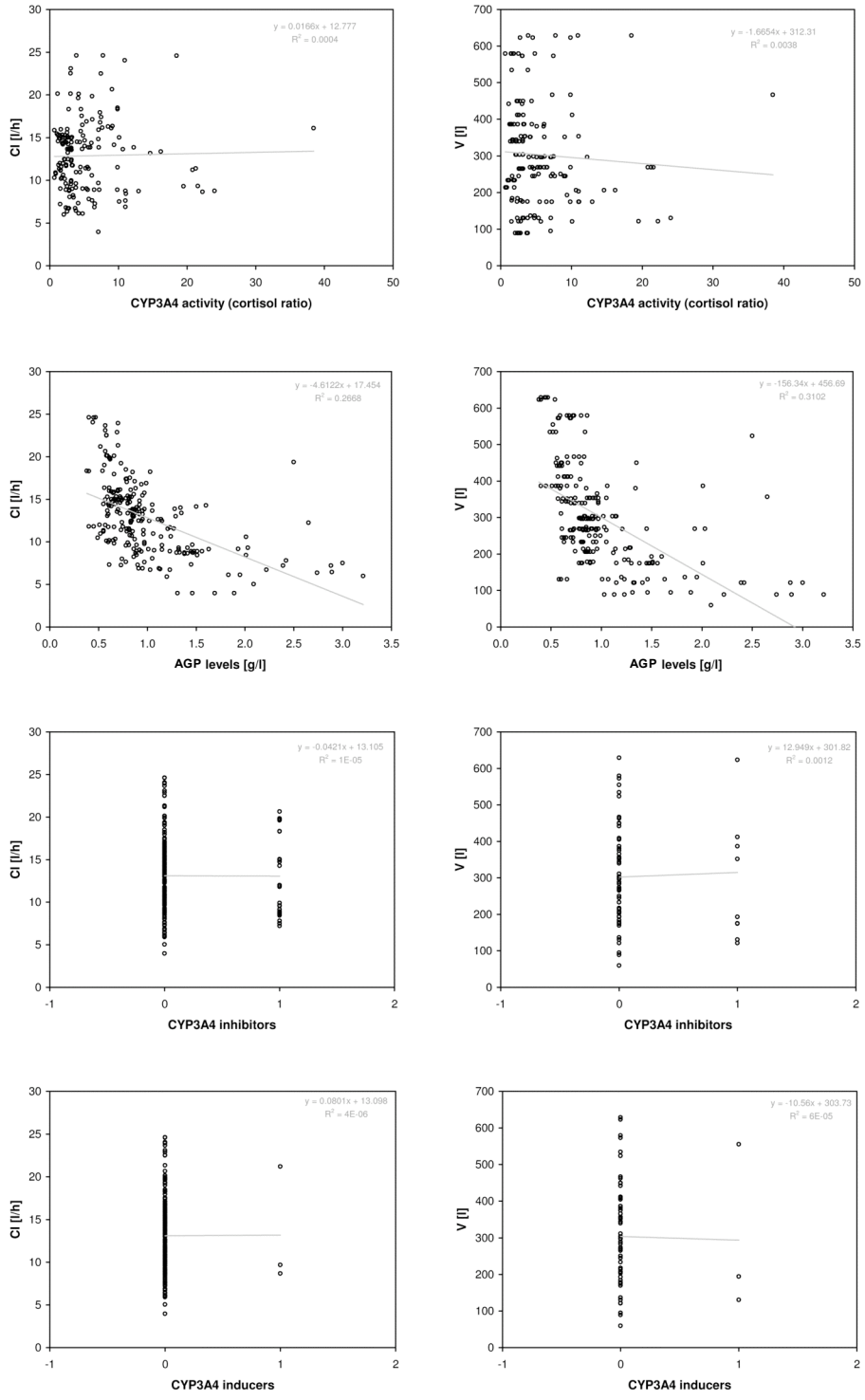
Appendix 3.5


Covariates examination with the final NONMEM model
y = 3.0274x + 11.354
R² = 0.1373
Cl [l/h]
Male
y = -50.359x + 332.66
R² = 0.0375
V [l]
Male
y = -0.1051x + 18.745
R² = 0.1493
Cl [l/h]
Age [year]
y = -2.2124x + 422.5
R² = 0.0653
V [l]
Age [year]
y = 0.196x - 20.296
R² = 0.2315
Cl [l/h]
Height [cm]
y = -1.5351x + 561.95
R² = 0.0141
V [l]
Height [cm]
y = 0.1168x + 4.6322
R² = 0.2116
Cl [l/h]
Body weight [kg]
y = -2.2918x + 469.79
R² = 0.0804
V [l]
Body weight [kg]

Covariates examination with the final NONMEM model



Covariates examination with the final NONMEM model



Appendix 3.6

The following table summarizes the development of the demographic model of imatinib population pharmacokinetics (FO method):

Hypothesis			Model	θ_a	θ_b	θ_c	θ_1	θ_2	θ_3	θ_4	ΔOF
Demographic covariates model (n=59)											
Basic model				12.1	299	0.467					-
Does BW influence	CL?		$\theta_a+\theta1\cdot BW$	12.1	255	0.433	13.5				-63.4
	Vd?		$\theta_b+\theta1\cdot BW$	12.1	299	0.466	0.00				+0.0
Does gender influence	CL?		$\theta_a+\theta_2\cdot q-\theta_2\cdot(1-q)$	12.2	267	0.433		2.3			-36.1
	Vd?		$\theta_b+\theta_2\cdot q-\theta_2\cdot(1-q)$	12.1	281	0.389		-77.9			-8.6
Does age influence	CL?		$\theta_a+\theta_3\cdot AGE$	12.3	307	0.470			-1.6		-1.6
	Vd?		$\theta_b+\theta_3\cdot AGE$	12.1	299	0.466			0.0		+0.0
Does pathology diagn. influence		CL?	$\theta_a+\theta_4\cdot p-\theta_4\cdot(1-p)$	12.8	295	0.442				-1.6	-9.9
		Vd?	$\theta_b+\theta_4\cdot p-\theta_4\cdot(1-p)$	12.1	255	0.372				53.1	-2.5
Does CYP3A4 inh. influence CL?			$\theta_a+\theta_6\cdot INH$	12.0	295	0.454					+0.9
Does CYP3A4 ind. influence CL?			$\theta_b+\theta_7\cdot IND$	12.1	299	0.467					+0.0
Do BW+gender influence CL?			$\theta_a+\theta_1\cdot BW+\theta_2\cdot q-\theta_2\cdot(1-q)$	12.1	259	0.451	11.2	0.8			-66.9
Do BW+gender+age influence CL?			$\theta_a+\theta_1\cdot BW+\theta_2\cdot q-\theta_2\cdot(1-q)+\theta_3\cdot AGE$	12.4	268	0.466	11.7	0.7	-2.2		-71.4
Do BW+gender+age+pathology diagn. influence CL?			$\theta_a+\theta_1\cdot BW+\theta_2\cdot q-\theta_2\cdot(1-q)+\theta_3\cdot AGE+\theta_4\cdot p-\theta_4\cdot(1-p)$	12.8	271	0.471	11.4	0.7	-1.7	-0.9	-76.0
Do BW+gender+age+pathology diagn. influence CL, and gender Vd?			**$\theta_a+\theta_1\cdot BW+\theta_2\cdot q-\theta_2\cdot(1-q)+\theta_3\cdot AGE+\theta_4\cdot p-\theta_4\cdot(1-p)$ $\theta_b+\theta_5\cdot q-\theta_5\cdot(1-q)$**	**12.8**	**258**	**0.437**	**12.7**	**0.8**	**-2.1**	**-1.0**	**-81.1**

$\theta_{a,b,c}$ = tested PK parameters (clearance, CL, volume of distribution, Vd and absorption constant, k_a, respectively); $\theta_{1,2,3,4,5,6,7}$ = covariate coefficient estimates.

BW (body weight) and age are expressed as the relative deviation of the individual BW and age from the population mean (70 kg and 50 years, respectively).

INH or IND = 1 if concomitant inhibitor/inducer drug is present; q = 0 if female and 1 if male; p = 0 if CML, 1 if GIST

n = number of patient in the data set.

ΔOF = difference in the NONMEM objective function (OF) compared to baseline model.

Appendix 3.7

The following table summarizes the models used to examine the influence of biological covariates on imatinib CL and Vd (FO method):

Hypothesis		Model	θ_a	θ_b	θ_c	θ_1	θ_2	θ_3	ΔOF
AGP model (n= 51)									
1-compartment, 1st order			11.8	322	0.660				-
Does AGP influence CL (eq. 6)?		CL = f(eq. 6)	12.5	308	0.820				-170.5
Does AGP influence CL (eq. 7)?		CL = f(eq. 7)	12.2	320	0.672				-5.6
Does AGP influence CL (eq. 8)?		CL = f(eq. 8)	12.2	356	0.797				-267.2
Does AGP influence CL (eq. 9)		CL = f(eq. 9)	12.1	365	0.767				-271.4
Does AGP influence CL (eq. 10-11)?		CL_u = f(eq. 10-11)	1200	355	0.741				-284.1
Does AGP influence Vd (eq. 6)?		CL_u = f(eq. 10-11), Vd =f(eq. 6)	1110	327	0.707				-295.8
Does AGP influence Vd (eq. 7)?		CL_u = f(eq. 10-11), Vd =f(eq. 7)	1230	354	0.742				-267.2
Does AGP influence Vd (eq. 8)?		CL_u = f(eq. 10-11), Vd =f(eq. 8)	1040	338	0.732				-298.0
Does AGP influence Vd (eq. 12)?		CL_u = f(eq. 10-11), Vd =f(eq. 12)	1290	355	0.740				-267.2
Does BW influence CL_u?		CL_u = f(eq. 10-11), $\theta_a+\theta_1 \cdot BW$,Vd = f(eq. 6)	1100	320	0.672	1000			-325.0
Do BW+gender influence CL_u?		CL_u = f(eq. 10-11), $\theta_a+\theta_1 \cdot BW$ $+\theta_2 \cdot q-\theta_2 \cdot (1-q)$, Vd = f(eq. 6)	1100	306	0.656	844	61		-327.2
Do BW influence CL_u and gender influence Vd?		CL_u = f(eq. 10-11), $\theta_a+\theta_1 \cdot BW$, Vd = f(eq. 6), $\theta_b+\theta_2 \cdot q-\theta_2 \cdot (1-q)$	1040	317	0.661	964	28		-325.6
Do BW+gender+age influence CL_u?		CL_u = f(eq. 10-11), $\theta_a+\theta_1 \cdot BW$ $+\theta_2 \cdot q-\theta_2 \cdot (1-q)+\theta_3 \cdot AGE$, Vd = f(eq. 6)	1110	305	0.640	858	51	-151	-328.6
Do BW+pathology diagn. influence	**CL_u?**	**CL_u = f(eq. 10-11), $\theta_a+\theta_1 \cdot BW$ $+\theta_2 \cdot p-\theta_2 \cdot (1-p)$, Vd = f(eq. 6)**	**1180**	**324**	**0.698**	**-120**			**-332.0**
	Vd?	CL_u = f(eq. 10-11), $\theta_a+\theta_1 \cdot BW$, Vd = f(eq. 6), $\theta_b+\theta_2 \cdot p-\theta_2 \cdot (1-p)$	905	313	0.654	-8			-325.7
MDR1 genotype (n=36)									
AGP model			1170	289	0.699				-
Does *MDR1* influence	CL_u?	$\theta_a+\theta_1 \cdot MDRT$	1240	297	0.709	-66			-1.3
	Vd?	$\theta_b+\theta_1 \cdot MDRT$	11240	344	0.705	-51			-1.0
CYP3A4 activity (n= 39)									
AGP model			1180	257	0.460				-
Does CYP3A4 influence	CL_u?	$\theta_a+\theta_2 \cdot 3A4A$	1220	256	0.455		13		-0.2
	Vd?	$\theta_b+\theta_3 \cdot 3A4A$	1090	278	0.489		11		-1.0
CRT clearance (n=47)									
AGP model			1120	311	0.695				-
Does CL_{CRT} influence	CL_u?	$\theta_a+\theta_3 \cdot CLCRT$	1160	306	0.692			336	-5.7
	Vd?	$\theta_b+\theta_4 \cdot CLCRT$	1300	316	0.702			168	-7.0

$\theta_{a,b,c}$ = tested PK parameters (clearance, CL, free clearance, CL_u, volume of distribution, Vd and absorption constant, k_a, respectively); $\theta_{1,2,3}$ = covariate estimates (BW, gender, age, or pathology diagnosis, or respectively MDRT, 3A4A and CLCRT).

BW (body weight), age, AGP (AGP plasma levels), 3A4A (CYP3A4 cortisol ratio), CLCRT (creatinine clearance): expressed as the relative deviation of the individual BW, AGE, AGP_{tot}, 3A4A and CRT from the population mean (70 kg, 50 years, 0.95 g/L, 5.4 and 75 mL/min respectively).

q = 0 if female, 1 if male; MDRT = -1 if 3435CC, 0 if 3435CT, 1 if 3435TT; p = 0 if CML, 1 if GIST

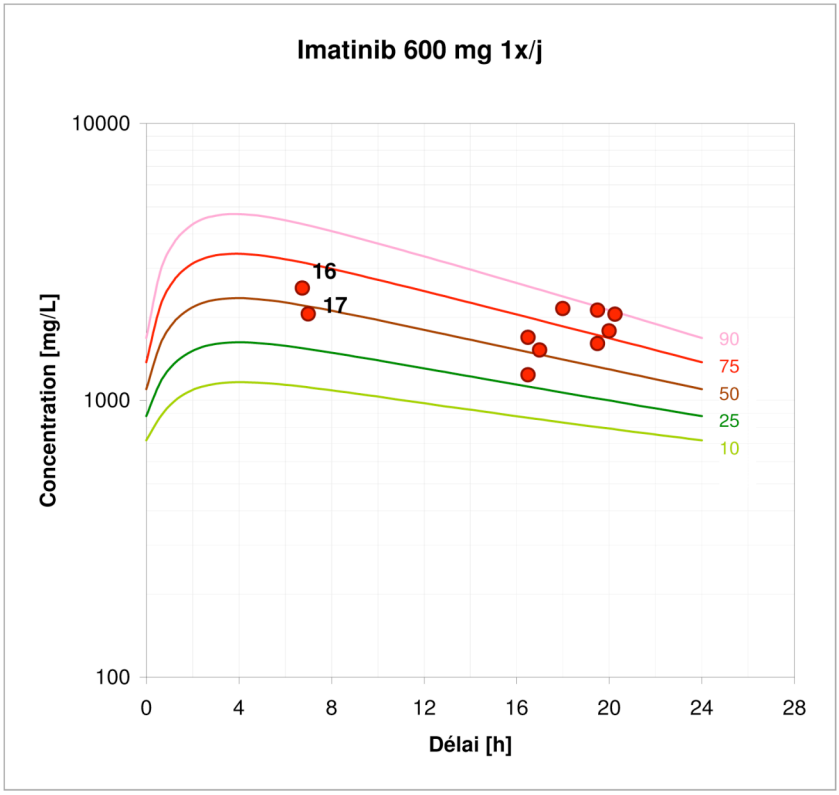
n = number of patient in the data set.

ΔOF = difference in the NONMEM objective function (OF) compared to baseline model.

In equation (12) the C_{tot} variable was defined as equal to the DV variable and was thus simply duplicated from this one.

Appendix 5.1

Représentation des percentiles de concentrations plasmatiques d'imatinib au cours d'un intervalle de dosage*



Patient: [redacted]

Taux	Date	Conc. [mg/L]	Délai [h]
7	24 juillet 2002	1780.8	20.00
8	26 septembre 2002	1605.1	19.50
9	9 janvier 2003	2116.1	19.50
10	10 avril 2003	2048.8	20.25
12	10 mars 2004	1237.0	16.50
13	2 juin 2004	1519.3	17.00
14	6 octobre 2004	1690.3	16.50
15	16 février 2005	2148.4	18.00
16	22 juin 2005	2547.4	6.75
17	19 octobre 2005	2054.1	7.00

* Ces courbes de percentiles ont été calculées à partir des données pharmacocinétiques obtenues à partir des dosages plasmatiques observés à l'état d'équilibre dans une population de 59 patients présentant une LMC ou un GIST